The role of the Coxsackie and Adenoviral Receptor in TNF alpha driven inflammation

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THE ROLE OF THE COXSACKIE AND ADENOVIRAL RECEPTOR IN TNF ALPHA DRIVEN INFLAMMATION

By

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Abstract

Transepithelial migration (TEpM) of leucocytes during the inflammatory process requires engagement with receptors expressed on the basolateral surface of the epithelium. One such receptor is Coxsackie and Adenovirus Receptor (CAR) which binds to Junction Adhesion Molecule - L (JAM-L) on leucocytes during TEpM.

This study reports the first evidence that TEpM of leucocyte cells requires, and is controlled by, phosphorylation of the cytoplasmic tail of CAR. The *in vitro* data shows that these leucocyte cells can adhere to an epithelial layer but where the cytoplasmic tail of CAR is prevented from undergoing phosphorylation the leucocytes are unable to transmigrate. Furthermore it shows that this CAR phosphorylation step is driven by TNF α signalling via a TNFR1-PI3K-PKCδ dependent signalling pathway. The work demonstrates that THP-1 cells can secrete TNF α thereby activating the CAR phosphorylation pathway leading to TEpM without addition of exogenous TNF α but importantly where TNF α is added this process is augmented suggesting a role for CAR in inflammatory conditions. Mouse models also confirm that CAR phosphorylation in response to inflammatory stimuli occur *in vivo*. Both acute (a 24 hour inhaled TNF α challenge) and chronic (a 34 day ovalbumin challenge) inflammatory conditions are studied. Confocal microscopy techniques are used to show that the cytoplasmic tail of CAR is phosphorylated. Specifically this is seen at the cell membrane of epithelial cells of bronchioles with associated inflammatory cells in the interstitium.

Taken together these data describe a novel method for the control of TEpM by transmigrating leucocytes that can also be heightened by the presence of pro-inflammatory cytokines during inflammation. This provides a novel target for controlling inflammation at the epithelium, a key component of the pathogenesis of many diseases including asthma.

Acknowledgements

I must start by expressing my gratitude to my two supervisors Professor George Santis and Dr Maddy Parsons. They have both worked incredibly hard to nurture me through the PhD. More significantly their enthusiasm and example has ensured that despite all the challenges that face the scientific community in the current economic climate I am left with a strong desire to continue with a career in medical research that could well have been lost without their involvement.

I would also like to thank Dr Penny Morton for the invaluable support I have received from her over many years both during the PhD and in the years leading up to it. Without her technical advice and assistance I would have floundered in the world of research many years ago but more importantly without her unwavering patience and reassurance when I repeatedly forgot the simplest steps in an experiment there would be no thesis now to review. As a result any weakness in my research knowledge and ability is no reflection on all the effort she has put in to train me.

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Finally and most importantly I must give a heartfelt thank you and apology to my wife, Vicki, for supporting me through the last few years. These have included many anxious and grumpy moments, with multiple weekends and evenings lost to work. Her understanding and support, with the aid of our ‘helpful’ cats Florence and Livingstone, have ensured that there is a completed body of work and a sane individual at the end of the process.

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Abbreviations

AACARGFP	Phospho dead CAR over expressing cell line
AJ	Adherens Junctions
Akt	Ak thymoma
APKC	Apical protein kinase C
ATII	Alveolar type 2 cell
Ca^{2+}	Calcium
CAR	Coxsackie and Adenovirus Receptor
CARGFP	CAR overexpressing cell line with a GFP tag
CD	Cluster of differentiation molecule
cIAPs	Cellular inhibitors of apoptosis
CLMP	CAR like membrane protein
CNS	Central nervous system
D1	Domain 1
D2	Domain 2
DAG D	Diacylglycerol
DDCARGFP	Phosphorylation mimic CAR overexpressing cell line
ECD	Extra cellular domain
EMTU	Epithelial mesenchymal trophic unit
ESAM	Endothelial Selective Adhesion Molecule
FADD	Fas-associated death domain protein
Fc receptor	Fragment, crystallisable region
FITC	Fluorescein isothiocyanate
GFP	Green florescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBEC	Human bronchial epithelial cells
hCD2	Human cluster of differentiation 2
hTERT	Human telomerase reverse transcriptase
ICAM-1	Intercellular Adhesion Molecule 1
Ig	Immunoglobulin

IHC	Immunohistochemistry
IL	Interleukin
INF	Interferon
IP	Immunoprecipitation
JAM	Junctional adhesional molecule
kDa	Kilo dalton
LFA1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 antigen
MAG1	Membrane-associated guanylate kinase 1
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney cells
mRNA	Messenger ribose nucleic acid
MUPP1	Multi-PDZ domain protein 1
NF-κB	Nuclear factor kappa beta
NK cells	Natural Killer cells
NMR	Nuclear magnetic resonance
nPKCδ	Novel protein kinase C delta
p120	protein 120
p21	Protein 21
PAR	Partitioning defective
PDBu	Phorbol 12,13-dibutyrate
PDZ	Post synaptic density protein/ Drosophila disc large tumour suppressor/ zonula occludens-1
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PICK1	Protein interacting with protein C kinase
PKC	Protein kinase C
PSD-95	Postsynaptic density 95
RIP1	Receptor interactin protein 1
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction

SHP 1 and 2	Src homology region 2 domain-containing phosphatase-1&2
SIRPα	Signal regulatory protein alpha
TEpM	Trans-epithelial migration
TH1	T –helper cell 1
TH2	T- helper cell 2
TJ	Tight Junctions
TNF α	Tumour necrosis factor alpha
TNFR1 & 2	TNF-receptor 1 & 2
TRAF2	TNF-receptor associated factor 2
VLA-4	Very Late Antigen-4
WB	Western blot
Wt	Wild type HBEC cell line
ZO	Zonular Occludins
γδT	Gamma delta T cells
µM	Micro meter

1 : Introduction

1.1 The Respiratory Epithelium

The respiratory epithelium comprises a layer of cells that line the respiratory system. It is predominantly made up of three epithelial cell types that work in combination to ensure the smooth flow of air in and out of the lungs and ensure that harmful substances do not enter the body. These cells include columnar epithelial cells, goblet cells and basal cells. In the oro and nasopharynx these columnar epithelial cells are replaced by squamous cells as they are designed to withstand the abrasive nature of the environment with an increased turnover of cells. As the layer reaches the alveolar space type I and II pneumocytes predominate to facilitate gas exchange. The epithelial cells form a single monolayer as they are all in contact with the underlying basement membrane but are referred to as 'pseudo-stratified' as in cross section the nuclei are not aligned and thereby the cells appear to be on top of one another. They do not function in isolation but instead interact closely with the underlying mesenchymal tissue, including dendritic cells and fibroblasts to affect the overall function of the airways. This process is key to both homeostasis of the lung airway and repair through stimulation of the primary stem cells within the lung epithelium: basal cells (cartilaginous airways), club cells (cartilaginous airways and bronchioles) and ATII cells (alveoli).

1.2 The Role of the Respiratory Epithelium in Asthma and Inflammation

The respiratory epithelium performs a complex role in the maintenance and function of the respiratory system. With an estimated area of $100m^2$ it provides the largest surface area in contact with the outside world (Holgate, 2007). With

each breath the airway is exposed to a variety of allergens and infectious agents. In view of this, it functions as a barrier, which incorporates physical, chemical and immunological actions (Lambrecht and Hammad, 2012, Davies, 2009, Tam *et al*., 2011). As such, the lung epithelium is in a central position to control immune homeostasis. Understanding of the immune system in the lungs has historically focused on leucocytes as both effectors and controllers. However an epithelial-centred approach provides an explanation for the lungs response to a diverse degree of stimuli including smoke, viruses and allergens (Hallstrand *et al*., 2014). This conclusion was first drawn following experiments that aimed to produce an asthma model (Boushey and Holtzman, 1985, Holtzman *et al*., 2014). The concept has developed as epithelial cells have been shown to have pattern recognition receptors that are designed to recognise foreign and potentially dangerous inhaled materials (Holgate *et al*., 2000, Lambrecht and Hammad, 2012). Asthma, as a disease entity, provides a paradigm of the growing evidence for the role of the epithelium in the inflammatory process. The term was originally used to describe patients with paroxysmal dyspnoea in 1860 by Henry Salter (Cohen, 1997). Our concept and treatment of asthma developed with an understanding of allergic pathways associated with atopy and the function of white blood cells and in particular eosinophils and more recently T lymphocytes in inflammation. However, despite our improved knowledge of the function of these cells *in vitro* and their responses, treatments have proved unsuccessful in controlling all aspects of the disease. This reflects its heterogeneous nature and the complex interaction that occurs *in vivo* between the individual and the environment which incorporates a variety of different cell types and has led to the introduction of the concept of the epithelial mesenchymal trophic unit (EMTU) (Holgate *et al*., 2000). This model places epithelial and mesenchymal cells in an

important bidirectional role in the inflammatory response seen in asthma as these cells both trigger a response to a stimuli and respond to other cells that are activated by the provocation (figure 1.1).

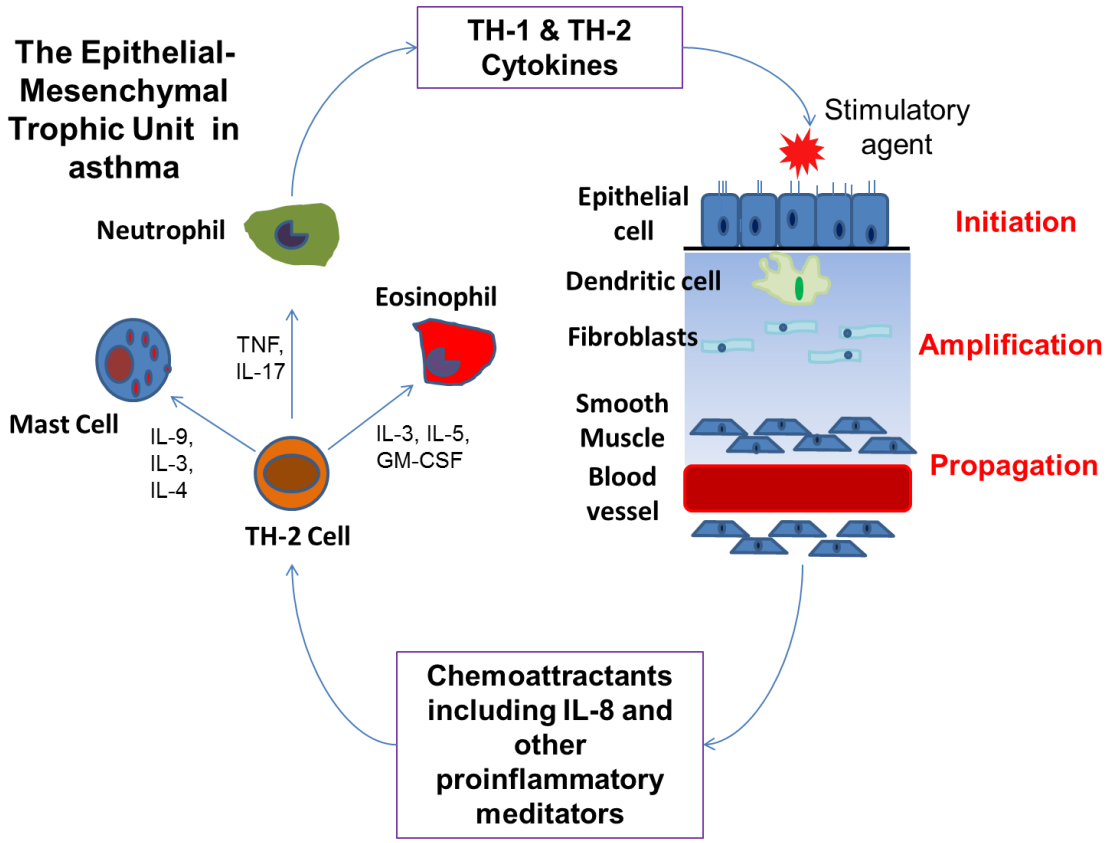


Figure 1-1: The Epithelial-Mesenchymal Trophic Unit.
Demonstrating the interaction between immunological and inflammatory mechanisms and structural elements of the respiratory system, placing epithelial cells in a key position to control the response. Adapted from Holgate 2010 (Holgate, 2010).

Aside from their ability to provide the initial response and stimulus to a perceived threat in the airway, epithelial cells are also key to controlling access either into or out of the airway thereby regulating the reaction that can occur. To perform its barrier role, the epithelium forms a continuous and highly regulated gate through which there can only be controlled movement. Interestingly, in a chronic lung inflammatory state in patients with asthma there is disruption of this barrier. This process has long been shown in asthmatic patients at a broad structural level

with the ‘desquamation’ of epithelial cells in post-mortem bronchoscopy specimens of patients dying as a result of their disease (Hogg, 1993). In life this disruption happens in a more subtle fashion through the interruption of stable links between cells with the loss of distinct junctional proteins (Holgate, 2007). This was demonstrated when cells taken from asthmatic patients and grown *in vitro*, for several passages, in the absence of other inflammatory mediators failed to form effective tight junctions with confocal staining demonstrating the reduction of the tight junction proteins Zonular Occludins-1 (ZO-1) and occludin (Holgate, 2007). Further work has supported this finding (Xiao *et al*., 2011) and additionally showed that adherens junctions are altered via a reduction in E-cadherin in patients with asthma (Lambrecht and Hammad, 2012). These data highlight the impact that the link between individual epithelial cells, as provided by their junctions, may play in during inflammation. Our understanding of the role of specific junctional proteins, including Coxsackie and Adenoviral Receptor, in inflammation and particularly asthma is reviewed in the following sections.

The model also shifts our understanding of the overall structural impact of the epithelium on airway remodelling in asthma, as the characteristic increase in smooth muscle surrounding the airway wall and thickening of the basal lamina can be seen in the relative absence of airway inflammation (Baraldo *et al*., 2011). This paradigm is instead dependent on abnormal wound healing driven by failure of the epithelial layer to regenerate appropriately leading to loss of its barrier integrity as a result of abnormal stem cell activation (Volckaert and De Langhe, 2014).

1.3 Epithelial Cell Junctions

The epithelium, as described, is composed of a single layer of epithelial cells. These cells are held in contact by a series of junctional complexes originally identified at an ultrastructural level in 1963 (Farquhar and Palade, 1963). They contain three different junctional structures (Figure 1-2). Adherens junctions form the initial cell to cell interaction through homotypic transmembrane adhesions. They are made up of two complexes; nectin-afadin complex and the classical cadherin-catenin complex, of which in the respiratory epithelium E-cadherin is the predominant cadherin present (Hallstrand *et al*., 2014, Niessen, 2007). These junctions are dynamic in nature to provide the cell contacts as they link to the cells internal actin cytoskeleton and microtubules via p120 and cytoplasmic adaptor proteins α-catenin and β-catenin (Hallstrand *et al*., 2014).

A second more apical link is formed by tight junctions (TJ's) whose primary function is to control the passage of solutes and immune cells between epithelial cells, either from the airway lumen into the interstitium or vice versa. These junctions are made up of a complex of transmembrane proteins that either homodimerise with proteins in adjacent cells or with alternative proteins on transmigrating cells. These proteins include members of the junctional adhesion molecule family (JAM's), claudins and occludins. They in turn link to ZO at the cell membrane to form a stable tight junction complex.

Finally desmosomes form a third interaction to resist mechanical stress and shearing forces on the epithelium. They consist of non-classical cadherins such as desmocollin and desmoglein which form strongly adhesive links between the filamentous cytoskeleton of epithelial cells and the lamina propria (Garrod and Chidgey, 2008).

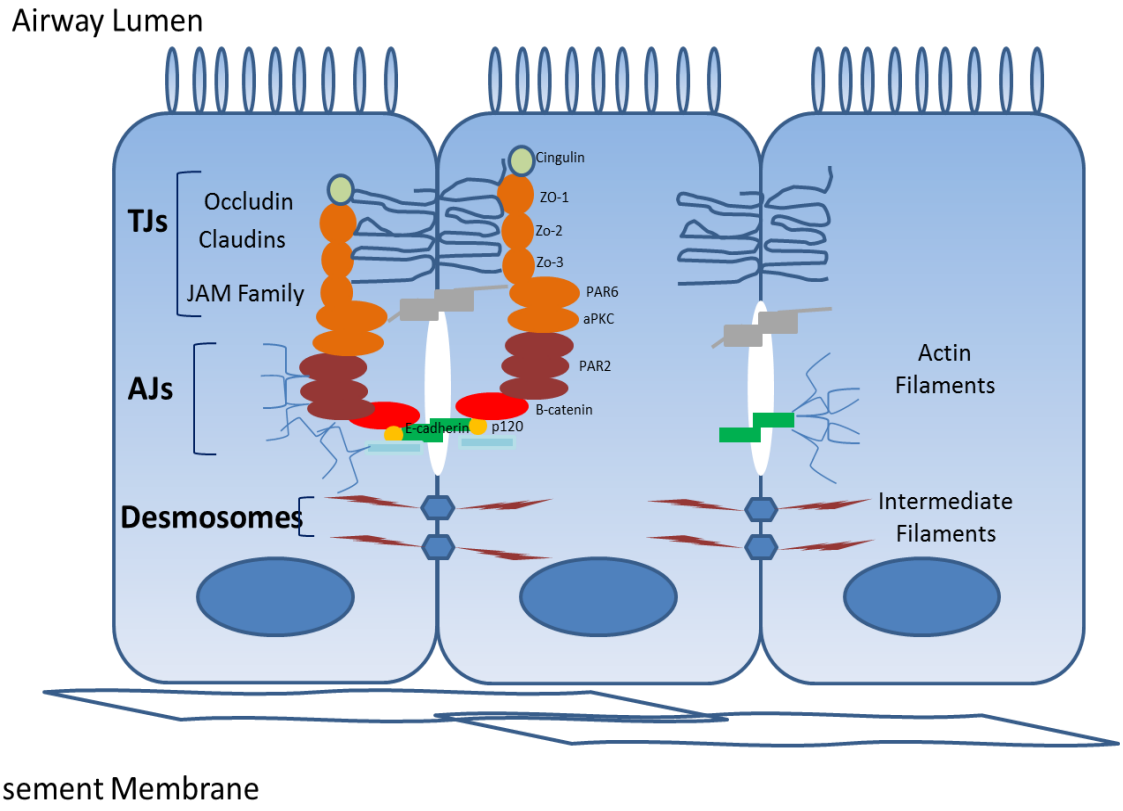


Figure 1-2: Cell to cell adhesion in the airway epithelium.
Showing the three common connections made between epithelial cells. Tights junctions shown are formed from the interaction between transmembrane proteins, Junction Adhesion Molecules (JAM'S), claudins and occludin anchored to further proteins including the Zonular Occludins (ZO) and Partitioning defective (PAR) proteins. They function to control permeability between cells. Adherens junctions mechanically connect adjacent cells and initiate the formation and maturation of cell–cell contacts through homotypic adhesions of E-cadherin, which is stabilized in the membrane by binding to anchor proteins p120 catenin, β-catenin, and α-catenin, which form an interface with the cell's microtubule network and actin cytoskeleton. Desmosomes consist of non-classical cadherins and form adhesive bonds with the filament cytoskeleton of the cells. Adapted from Hallstrand et.al (Hallstrand *et al*., 2014).

1.4 Tight Junctions

Tight junctions, as described, provide discrete contacts between adjacent cells that enable them to control paracellular permeability of water, solutes and cells as well as serving to define the apical and basolateral membrane compartments of polarised epithelial cells (Cohen *et al*., 2001, Anderson and Van Itallie, 2009). This mechanism is vital in the normal physiological function of the airway epithelial layer as it is key to providing an interface between the external

environment and the underlying tissue (Swindle *et al*., 2009). Where this is disrupted there is uninhibited movement of anything from small molecules to cells. This process is bi-directional as immune cells can pass out into the lumen.

Tight junctions are formed by the interaction of multiple transmembrane receptors including occludin, claudins, tricellulin and the JAM family, of which Coxsackie and Adenovirus Receptor (CAR) is a member (figure 1.3) (Schulzke and Fromm, 2009). Tight junction associated adaptor proteins, such as the ZO family, link the membrane to the actin cytoskeleton. Together they work in complex to stabilise both endothelial and epithelial layers and to control passage of substances between cells (Hardyman *et al*., 2013). They sit at the most apical point of the intracellular junctions and as such mark the boundary between the apical membrane of the cells and the basolateral component (Niessen, 2007). When formed they pull the two adjacent cell membranes very closely together to ensure there is almost no intracellular space through which solutes can pass uncontrolled. In addition to this barrier function, specific tight junctions components can play individual roles such as receptors, signalling molecules or regulators of absorption (Schulzke and Fromm, 2009).

The proteins that make up the tight junction can be modified in multiple ways to alter the function and structure of the junction. This includes steps to control the presence of the protein at the junction through regulation of gene expression, modification of mRNA or protein half-life, endocytosis and/or micropinocytosis and cleavage of tight junction proteins (Schulzke and Fromm, 2009). Alterations can also be made to the activity of the proteins when they are at the junctions via phosphorylation (Gonzalez-Mariscal *et al*., 2008). The importance of phosphorylation of tight junction proteins was first advanced through its impact on ZO-1 which when phosphorylated was found be associated with a loss of trans-

epithelial resistance (Stevenson *et al*., 1989). Our understanding of the relevance of these phosphorylation events is hampered by the fact that individual tight junction proteins can be phosphorylated at different sites by different kinases at the same time. One such family of kinases is the Protein kinase C (PKC) family which comprises 15 separate enzymes subdivided into three separate groups depending on their method of activation: (1) conventional (α, β1, β2 and γ) which are Ca^{2+} and diacylglycerol (DAG) dependent, (2) novel (δ, ε, θ, η and μ) which are DAG dependent and (3) atypical (λ, ξ and τ) which are both Ca^{2+} and DAG independent.

These kinases phosphorylate serine/threonine residues and have shown to be active at tight junctions following a number of diverse stimuli including oxidative stress (Perez *et al*., 2006), calcium wash out (Citi, 1992), vascular endothelial growth factor (Muto *et al*., 2000) and cytokines (Tumour necrosis factor (TNF α) and Interferon γ) (Coyne *et al*., 2002). Interestingly they have been shown to phosphorylate specific tight junction proteins causing a variety of disease states. For example when nPKCδ is blocked, occludin is no longer phosphorylated with a resultant loss of tight junction formation in MDCK cells (Andreeva *et al*., 2006). Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) is another kinase associated with tight junction protein phosphorylation. It works in sequence with Akt to drive serine/ threonine phosphorylation. Depending on the cell type and stimulus used its activity can lead to both an increase and a decrease in junction stability (Gonzalez-Mariscal *et al*., 2008). Therefore proteins in the tight junction are not in a passive state but instead can be controlled by the overall epithelial state.

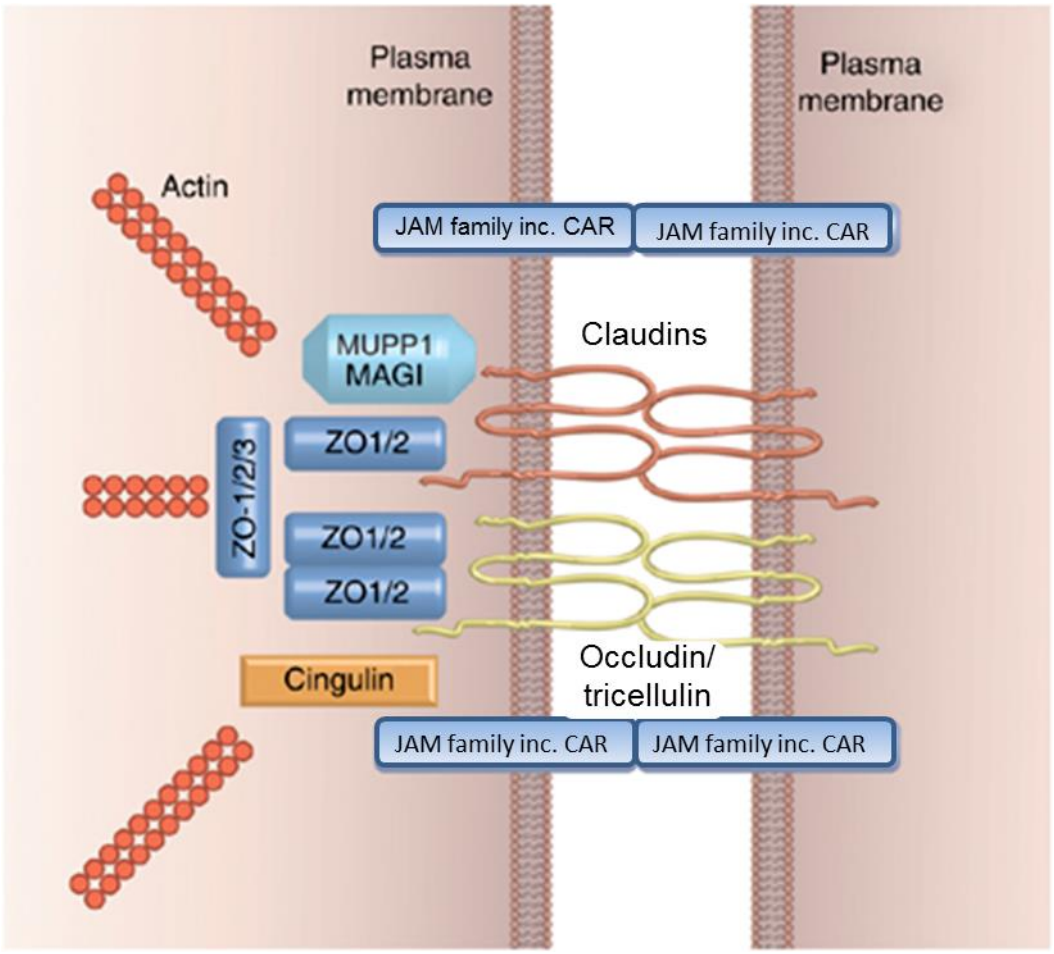


Figure 1-3: Graphic representation of a tight junction.
Includes the transmembrane components comprising claudins, occludin, tricellulin and JAM's. Also shown are the intracellular anchoring proteins (ZO1/2, Membrane-associated guanylate kinase 1(MAGI), Multi-PDZ domain protein 1(MUPPI), Cingulin) necessary to provide a link to the actin cytoskeleton adapted from Niessen 2007 (Niessen, 2007).

1.5 Junction Adhesion Molecules

The Junction Adhesion Molecule (JAM) family make up part of the tight junction complex and are members of the larger immunoglobulin (Ig) superfamily of adhesion receptors. They are part of the same immunoglobulin family due to their shared extracellular Ig-like domains: a membrane-distal V type Ig-domain and a membrane-proximal C2-type Ig-domain that allow for both homo and heterodimerisation *in trans* (Ebnet *et al*., 2004). Within the family itself there is a degree of additional grouping based on their amino acid sequence. The JAM

family include a distinct group sometimes referred to as the 'classical' JAM proteins with an approximate 35% amino acid similarity containing JAM-A (Malergue *et al*., 1998), JAM-B (Cunningham *et al*., 2000, Palmeri *et al*., 2000) and JAM-C (Arrate *et al*., 2001). These three JAM family members are distinct as they contain a class II PDZ-binding motif at the C-terminus of their cytoplasmic tail (Ebnet *et al*., 2004). Coxsackie and Adenovirus Receptor (CAR), CAR like membrane protein (CLMP), Endothelial Selective Adhesion Molecule (ESAM) and JAM-4 make up a separate subfamily as they have an alternative class I PDZ-binding domain at the C-terminus of their cytoplasmic tail. This difference leads to alternative intracellular binding proteins for the different family members which in turn results in their different intracellular functions. The different subfamilies also vary by the average length of their cytoplasmic tails (figure 1.4). The variation in the tail length with the resultant presence of different phosphorylation sites suggests that these differences are present in order to enable the protein to cause functional changes to the cells.

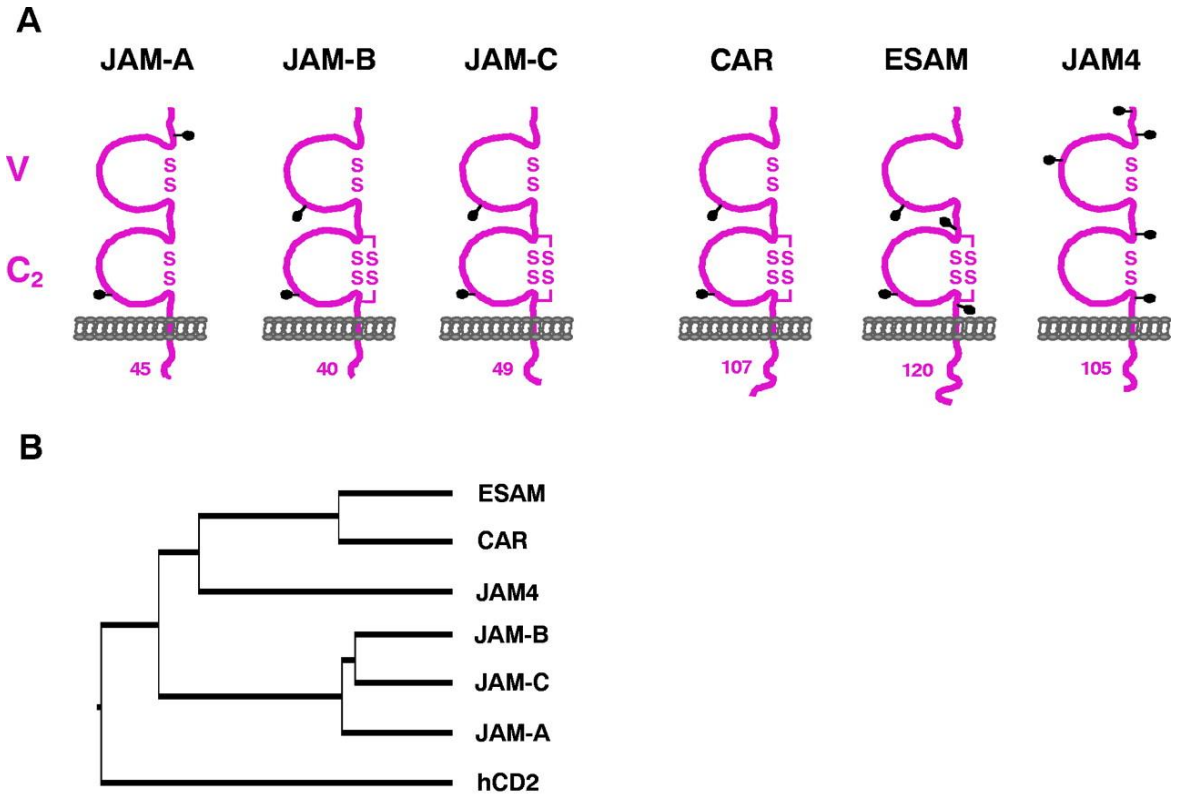


Figure 1-4: Members of the junction adhesion family of transmembrane proteins.
A – Showing different members of the JAM family and highlighting the variation in the cytoplasmic tail length. B – The phylogenic tree of for the JAM family (Ebnet *et al*., 2004).

Importantly although they do subtly differ in their amino acid structure and sequence their extracellular domains are capable of interacting. Individual JAM family proteins can form dimers with each other at the cell membrane. JAM-A for example was originally shown to be orientated in a U-shaped *cis* dimer at the cell membrane of individual endothelial cells which can homodimerise *in trans* with JAM-A found at the cell membrane in adjacent cells (Kostrewa *et al*., 2001). CAR is another member of the family to have been shown to homodimerise *in trans* at the cell membrane (Coyne and Bergelson, 2005) but interestingly it can also form heterodimers with other members of the JAM family, in particular JAM-L found on transmigrating leucocytes (Verdino *et al*., 2010, Witherden *et al*., 2010).

Significantly JAM-L expression is restricted to neutrophils, monocytes, and memory T cells (Luissint et al., 2008). At the cell membrane JAM-L interacts with VLA-4, where VLA-4 controls its dimerisation with CAR. VLA-4 is important in the transmigration of leukocytes through the endothelial layer. This interaction between CAR and JAM-L has an exceptional number of interdigitating salt bridges formed through the interaction of the domain 1 (D1) immunoglobulin components of both the CAR and JAM-L molecules which contributes binding energy but, more importantly, imparts high ligand specificity. CAR and JAM-L are not specific in their ability to form heterodimers as demonstrated in figure 1.5. Their specificity in forming heterodimers, therefore, places the JAM family in an important position to facilitate trans-epithelial migration (TEpM).

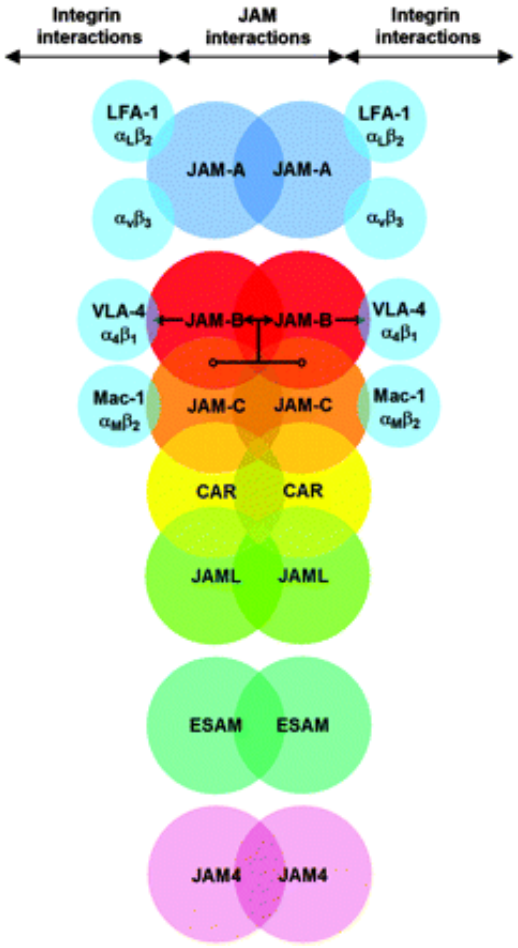


Figure 1-5: JAM family interactions.
The multiple interactions of JAMs. JAMs can form specific *cis*- or *trans*-interactions with other JAM members (area marked as JAM interactions). All JAM members have been described as capable of forming or potentially forming homophilic *cis* and *trans* interactions (overlapping same colour). Heterophilic *cis*- and *trans*- interactions can occur between specific members of the JAM family (interlaced colour) or *trans*-interactions with integrin partners (overlapping colour, area marked as integrin interactions) (Lymphocyte function-associated antigen 1(LFA-1), Very Late Antigen-4 (VLA-4) Macrophage-1 antigen (Mac-1). The prerequisite of a JAM-B/JAM-C interaction before JAM-B can engage VLA-4 is represented by arrows showing the sequential steps.(Bradfield *et al*., 2007)

Members of the JAM family are found in leucocytes, endothelial cells and epithelial cells (Luissint *et al*., 2014). Given their association with endothelial cells and leucocytes extensive research has been undertaken showing their importance to vascular permeability through barrier control and leucocyte cell trafficking (Garrido-Urbani *et al*., 2008, Bradfield *et al*., 2007). Although present

on epithelial cells too their role in trans-epithelial migration (TEpM) has been less well studied.

1.6 Coxsackie and Adenovirus Receptor

Coxsackie and Adenovirus Receptor (CAR) is a 46 KDa transmembrane protein that in its predominant isoform, found at the cell membrane, is made up of 346 amino acids (Coyne and Bergelson, 2005). It acts as a receptor for Coxsackie and Adenoviruses in addition to playing a role in cell-cell adhesion.

CAR was initially identified as the primary docking protein for Coxsackie B viruses (CV1-CVB6) and members of the adenovirus family (subgroups A, C, D, E and F but not B) (Bergelson *et al*., 1997, Tomko *et al*., 1997). It has been extensively studied as adenoviruses have been evaluated as candidate vectors in gene therapy for conditions such as cystic fibrosis (Kremer and Perricaudet, 1995) and in the treatment of a variety of cancers (Bruning and Runnebaum, 2003). The adenovirus CAR binding site is the same used for extracellular D1-D1 homotopic interactions and for heterodimerisation with JAM-L suggesting a reason for the sites preservation and use by viruses(Schreiber *et al*., 2014).

1.6.1 The Structure of CAR

Its structure comprises an extracellular domain, a transmembrane section and a cytoplasmic component (figure 1.6). As with other members of the JAM family, the extracellular domain is made up of two immunoglobulin components: a V-type Ig domain and a membrane-proximal C2-type Ig domain, referred to as D1 and D2 respectively and contains glycosylation and palmitoylation sites which are thought to be important in its function in Adenovirus infection (Coyne and Bergelson, 2005). The D1 component containing 118 amino acids has been expressed in bacteria and its crystalline structure forms a β-pleated sheet

sandwich fold that places it in the Ig family of membrane bound proteins (van Raaij *et al*., 2000, Bewley *et al*., 1999). Using Nuclear magnetic resonance (NMR) spectroscopy, the D2 component containing 98 amino acids has also been shown to form a β-pleated sheet sandwich in keeping with other members of the Ig superfamily but its sequence and structure does differ from other members with two β-sheets, one consisting of β-strands A, B and G and the other of β-strands C, E and G (Jiang and Caffrey, 2007).

There is also a single 23 amino acid trans-membrane domain which in the full length splice variant links to a 107 amino acid cytoplasmic domain (Coyne and Bergelson, 2005). There are splicing variants of the cytoplasmic tail that can result in an alteration in its structure and function. In human cells the gene responsible for CAR is found on chromosome 21q11.2 (Bowles *et al*., 1999). There have been several splice variants identified for the murine form of this gene, which can lead to alterations in the cytoplasmic tail (Chen *et al*., 2003). In Bergelson *et al's* initial description of human CAR, it is reported as a 7 exon gene (Bergelson *et al*., 1997). The same group identified a mouse version as an 8 exon gene (Bergelson *et al*., 1998). It has since been established that multiple isoforms of CAR exist with small variations of the 8 exons identified (Excoffon *et al*., 2010, Shaw *et al*., 2004, Raschperger *et al*., 2006, Gye *et al*., 2011). These isoforms are almost entirely identical but can have subtle physiological effects. For example; Excoffon *et al* showed that those forms that retain the terminal end of CAR's cytoplasmic tail and therefore have the PDZ binding component but are shortened by 13 amino acids in the 7 exon form compared to the 8 exon have a different membrane localisation. The exon 7 form is instead predominantly basolateral whereas the exon 8 form appears mainly apically (Excoffon *et al*., 2010) This alteration has been suggested as necessary for Coxsackie and

Adenoviruses infection of epithelial cells as the basolateral localisation of the originally identified exon 7 human form of CAR means that the receptor is hidden to a virus at the apical surface. However, across species the cytoplasmic tail is more extensively conserved than in other parts of the protein. For example, mice share 95% of the same amino acids on their cytoplasmic tail with the human version of CAR. The Zebrafish form of CAR is 44% identical to that found in human cells but this figure rises to 66% for the cytoplasmic tail suggesting that the tail plays an important physiological role thereby ensuring its evolutionary maintenance (Coyne and Bergelson, 2005).

Figure 1-6: Showing the four domains of CAR as a transmembrane protein (Coyne and Bergelson, 2005).

D1 and D2 are extracellular domains, followed by a transmembrane region and long cytoplasmic tail (c).

The intracellular section contains sites at which phosphorylation or palmitoylation can occur which suggests that it may play a physiological role in cell signalling and function (van't Hof and Crystal, 2002, Coyne and Bergelson, 2005). CAR truncation mutants have been used to show that the amino acids from 261 to 315 (which contain the tyrosine/threonine/serine phosphorylation sites in figure 1.7)

are required to both enable calyculin-driven phosphorylation of CAR and activate p44/42 in response to homodimerisation (Morton *et al*., 2013, Farmer *et al*., 2009). Furthermore two serine/threonine (290/293) phosphorylation sites have been specifically identified and have been shown to influence CAR meditated endocytosis of E-cadherin (Morton *et al*., 2013). In addition to this, the cytoplasmic tail contains one postulated tyrosine phosphorylation sites although to date this remains unvalidated (figure 1.7). There are also two membrane-proximal cysteines that can be subject to fatty acid acylation and a C-terminal hydrophobic peptide motif that interacts with PDZ-domain proteins (Tomko *et al*., 1997, van't Hof and Crystal, 2002). This C-terminal hydrophobic peptide is important in CAR's function at the cell membrane as it enables it to bind to multiple other structural proteins including ZO-1, membrane-associated guanylate kinase 1b (MAGI-1b), protein interacting with protein C kinase (PICK1) and postsynaptic density 95 (PSD-95) which implies that it is present in multiple protein complexes (Excoffon *et al*., 2010).

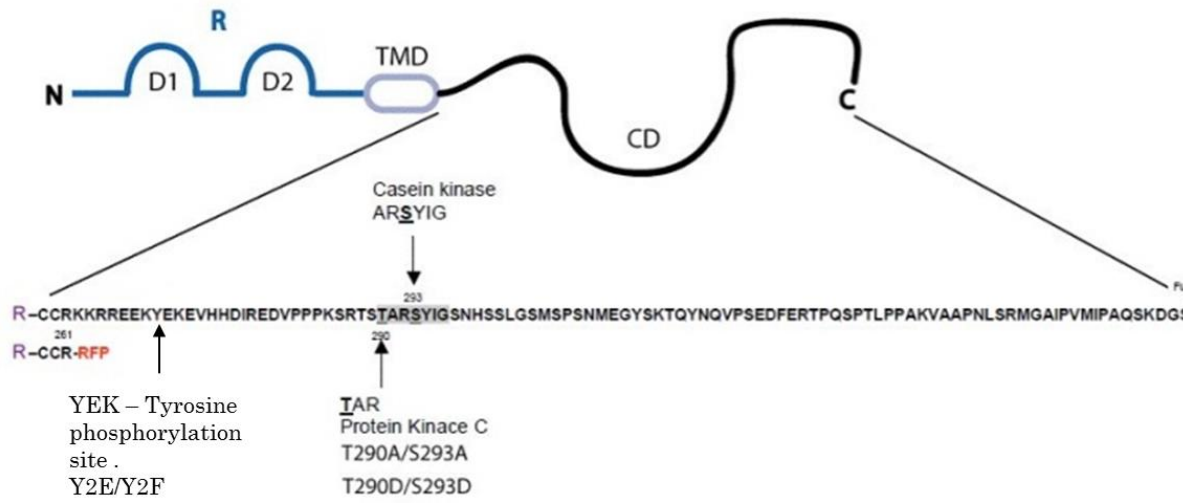


Figure 1-7: Possible sites for phosphorylation of the CAR cytoplasmic tail. Threonine -290 (TAR) and Serine – 293 (ARSYIG) sites have been shown to be phosphorylated previously (Morton *et al*., 2013). The tyrosine 261 site (YEK) is a putative site for phosphorylation. Key: CD – cytoplasmic domain, TMD – Transmembrane domain, D1 & D2 – extracellular domains.

1.6.2 The expression of CAR in the body

CAR expression varies between organs. Challenges with the available antibodies has limited detailed tissue staining, although Tomoko *et al* have shown high CAR expression levels in the liver, intestines and lung of rats, along with scattered signalling in the pancreas and heart. Interestingly within the lung the staining was seen in the epithelium of the trachea and bronchi but not alveoli (Tomko *et al*., 2000). Other work using Ribonucleic acid (RNA) blot analysis in humans suggests the highest expression levels are found in the heart, pancreas, brain, testis and prostate (Coyne and Bergelson, 2005, Bergelson *et al*., 1998). In these organs in mice, Raschperger *et al* showed that CAR was only localised to epithelial layers (Raschperger *et al*., 2006). However, in human skin derived cultured lymphatic endothelial cells, CAR expression is seen and is suggested to play a role in maintaining lymphatic vessel integrity (Vigl *et al*., 2009). These expression levels change during embryonic development with high levels

particularly present in the central nervous system and heart (Ito *et al*., 2000, Hotta *et al*., 2003). The embryonic expression in the developing heart has been shown to be particularly important by Chen *et al* where CAR deletion leads to mouse embryo death as a result of cardiac defects (Chen *et al*., 2006, Dorner *et al*., 2005).

In other tissue types CAR expression has been reported to be low or absent, including in healthy adult skeletal muscle (Fechner *et al*., 2003), primary human fibroblasts (Hidaka *et al*., 1999) and most peripheral blood cells (Huang *et al*., 1997), 1997) thereby ensuring they are difficult to transfect using adenoviral vectors.

Expression levels are also altered in response to different conditions as well as being tissue specific. In response to inflammatory conditions in the presence of TNF α and Interferon (INF) γ CAR expression levels are downgraded at the cell surface after 24 hours and to a greater degree at 48 hours in vascular endothelial cells (Vincent *et al*., 2004). Interestingly this response was not reproduced in either human bronchial epithelial cells or A549 cell line once again highlighting the varying response of CAR in different tissue and cell types.

1.6.3 CAR in tight junctions

The cellular role of CAR in the dynamic control and regulation of epithelial cell junction formation and stability has only been recently investigated (Cohen *et al*., 2001, Honda *et al*., 2000). It importantly plays this role at cell-cell junctions through homodimerisation *in trans* with the extracellular D1 domain of other CAR proteins in adjacent cells (figure 1.7). This is supported by the structural analysis of CAR D1 domain. The D1 component of CAR is able to form homodimers in the crystal lattice but also in solution, with a measured dissociation constant of 16 μM

(van Raaij *et al*., 2000) consistent with those measured for other cell adhesion complexes (van der Merwe and Barclay, 1994, van der Merwe *et al*., 1994).

Disruption of this extracellular interaction has been extensively explored in relation to adenovirus binding (Santis *et al*., 1999, Kirby *et al*., 2000, Walters *et al*., 2002). Notably this work has shown that the adenovirus type 5 fibre knob domain binds to CAR with much higher affinity than CAR binds to itself (Lortat-Jacob *et al*., 2001). This process therefore leads to loss of CAR homodimerisation at tight junctions. However, whether this homodimerisation is necessary for the intracellular functions of CAR via its cytoplasmic tail has not been established.

Where CAR is disrupted in its role as a tight junction protein by the addition of its soluble extracellular domain (CAR-ECD) to cell media, the trans-epithelial resistance is reduced and the rate of FITC-dextran passage increased (Cohen *et al*., 2001). These effects are organ specific, though, with loss of CAR at tight junctions leading to increased permeability in the heart but not in the gut of mice. (Pazirandeh *et al*., 2011) Aside from its direct role in tight junctions, CAR expression also effects adherens junction regulation. E-Cadherin levels, which play an integral role in respiratory epithelial cells, are reduced at these junctions in the presence of high levels of CAR (Morton *et al*., 2013). This event has been suggested as cause of the increased level of tumour activity seen with CAR positive tumours (Luissint *et al*., 2014). The explanation for this is that E-Cadherin associates with the transcription factor β–catenin. This means that when E-Cadherin is replaced at junctions β–catenin is free to translocate to the nucleus resulting in increased cell proliferation. This is supported by evidence from CAR knockdown cancer models where α–catenin is downregulated (Stecker *et al*., 2009). Its significance in tumourgenesis is, however, not universal as *in vivo* data

has proved contradictory. In the lungs a study of 120 lung cancer patients found that both by real time-PCR(RT-PCR) and western blot analysis the expression levels of CAR were higher in tumour cells and more specifically in patients with a squamous cell carcinoma their CAR expression levels equated to the tumour grade (Chen *et al*., 2013). This was supported by another study of multiple different tumour types that suggested CAR expression was raised in malignancy (Reeh *et al*., 2013). In other primary tumours and tumour cell lines, CAR expression has instead been seen to inversely correlate with the rate of cell proliferation, suggesting that CAR may act as a tumour suppressor (Fuxe *et al*., 2003, Okegawa *et al*., 2000). Induced expression of CAR has been shown to inhibit tumour cell growth in human prostate cancer (Okegawa *et al*., 2000, Rauen *et al*., 2002), bladder cancer (Li *et al*., 1999) and glioma cell lines (Kim *et al*., 2003), indicating CAR tumour inhibitory properties. Other reports also showed that absent or reduced expression of CAR is associated with a higher tumour grade in human prostate and bladder cancer patients, while healthy tissues express easily detectable CAR (Okegawa *et al*., 2001, Rauen *et al*., 2002). These earlier papers concentrated on CAR in the context of determining the success of possible gene therapy delivered by adenoviruses. This difference is reflected in the fact that they predominantly used cell lines rather than tissue samples which may explain the different clinical picture seen in later studies. Interestingly though, a possible explanation for CAR acting as a tumour suppressor is that the palmitoylation motif localised on the cytoplasmic tail of CAR appears to be essential for tumour-inhibitory activity and cell cycle regulation possibly via the downstream activation of p21 (Okegawa *et al*., 2001). This again highlights the possible importance of the cytoplasmic tail in the function of CAR. As previously discussed the serine/threonine phosphorylation status of the CAR cytoplasmic tail

effects the localisation of E-Cadherin to the cell membrane (Morton *et al*., 2013). Importantly E-Cadherin at the cell membrane has been found to be diminished in patients in the chronic inflammatory condition, asthma, suggesting a possible interaction between tight junctions and adherens junctions in long term inflammatory states (de Boer *et al*., 2008).

1.6.4 CAR in Inflammation

The role of CAR during inflammation is yet to be defined with conflicting data suggesting that its response is dependent on cell and tissue type. In rat cardiomyocyte cells expression is upregulated in chronic autoimmune inflammatory conditions using an experimental model based on the presence of porcine myosin (Ito *et al*., 2000). However, in the presence of a combination of cytokines including TNF and INF there was a reduction in CAR expression in human umbilical endothelial cells (Vincent *et al*., 2004). Interestingly this group did not show the same effect in a respiratory epithelial cell line. Therefore the presence of CAR during inflammation depends on a number of factors including both the source of the stimulus and the type cells being affected.

A mechanism for the role of CAR in this immune reaction has been suggested through more recent work that addressed CAR's interaction with other members of the JAM family (Verdino and Wilson, 2011, Witherden *et al*., 2010, Zen *et al*., 2005). As described previously CAR has a similar structure to other proteins in the JAM family and their extracellular components can heterodimerise. Some members of the family including JAM-A is also found on leucocytes as well as endothelial and epithelial cells. JAM-A disruption in leucocytes leads to a reduction in leucocyte trans-endothelial migration due to loss of homodimerisation with JAM-A found on epithelial cells (Martin-Padura *et al*., 1998). The more recent work by Verdino *et. al*. and Witherden *et. al*. showed that

CAR on the skin epithelial cell membrane binds to JAM-L on γδ T cells (Witherden *et al*., 2010, Verdino *et al*., 2010) and Zen *et al.* have shown that the same binding occurs between CAR on gut epithelial cells with JAM-L on neutrophils (Zen *et al*., 2005). This is important as these neutrophils and T cells play a key role in host immunity as they are found in the gut and skin, where they protect against environmental insults such infection, trauma and malignancy. Where this interaction between CAR and JAM-L is disrupted skin healing is slowed (Witherden *et al*., 2010). Therefore JAM-L, with its ligand CAR, can been seen as a co-stimulatory receptor for γδ T cells (Verdino and Wilson, 2011) which places CAR in the position to modulate the immune response.

Although as yet not fully understood, this complex interaction between epithelial cells and leucocytes can lead to disease and also therefore open the possibility for pathogenic modification.

1.7 Leucocytes and Trans-epithelial Migration (TEpM)

The fundamental function of the inflammatory process in response to infection or an allergen is the elimination and removal of the offending agent. For this to occur, the immune system needs to ensure its effector cells are able to reach the correct site. In the case of the lungs this is a multi-step process that requires the leucocytes to leave the vascular system by crossing the endothelial barrier, then move through the tissue in an appropriately targeted fashion, and finally cross through the epithelial barrier into the airway (Liu *et al*., 2004b, Garrido-Urbani *et al*., 2008, Klesney-Tait *et al*., 2013). As outlined in the previous section, CAR has been shown to play role in this process through its binding to proteins on transmigrating leucocytes.

There is evidence that CAR is firstly required in the movement of immune cells across the endothelium and into the interstitium. At the cell membrane JAM-L is known to immunoprecipitate with VLA-4 and this interaction is required for its dimerisation with CAR (Luissint et al., 2008). VLA-4 is also known to be important in the transmigration of leukocytes through the endothelial layer via its interaction with VCAM-1 thereby incorporating these molecules in a larger complex required for the transmigration process. This study does not focus on the process of trans-endothelial migration and the role of CAR in this but it does suggest that at the broader significance of any treatment directed at the CAR protein. By inhibiting CAR function at tight junctions immune cells would firstly by prevented from leaving the interstitium into the airway lumen but they would also be prevented from leaving the vasculature thereby preventing the a prolonged and protracted inflammatory response.

The process of TEpM requires a specific sequence of events to occur that is distinct although similar to trans-endothelial transmigration. The process is firstly different as the leucocytes approach the epithelial barrier from the basal surface which exposes them to a different selection of adhesion molecules than on the apical endothelial surface. Secondly, the leucocytes themselves have been given an extended period of priming by their passage through the endothelial barrier and the surrounding tissue, during which they will be exposed to both chemokines and cytokines thereby altering their state of activation. Finally the actual distance they need to travel when crossing the epithelial barrier is significantly larger than the endothelium. There is a minimum 20µm additional distance to traverse with the resultant need for greater interaction between the leucocyte and epithelial cell.

The first step in TEpM requires the adhesion of leucocytes to the basal membrane of the epithelial cell. In neutrophils this adhesion step is critically controlled by β2 integrins and in particular CD11b/CD18 on the neutrophil (Zemans *et al*., 2009, Parkos *et al*., 1991). This adhesion step has most extensively been studied in intestinal epithelial models but has also been shown to occur in the respiratory epithelium (Celi *et al*., 1999, Jagels *et al*., 1999). This effect has been confirmed through antibody blocking experiments whereby competitive blocking of CD11B/CD18 leads to loss of neutrophil transmigration (Parkos *et al*., 1991). Furthermore the binding action is stimulated by the presence of TNF α highlighting the importance of the interplay between cytokines and junctional proteins in leucocyte transmigration (Miyata *et al*., 1999). Specifically in the lungs the β1 integrin (CD29) also appears to act as an adhesion molecule as the presence of CD29 blocking antibodies in lipopolysaccharide (LPS) induced inflammatory conditions led to a reduction in the accumulation in neutrophils in the mouse airway (Ridger *et al*., 2001). The corresponding binding component on the epithelial cell does differ from that on endothelial cells. Intercellular Adhesion Molecule 1 (ICAM-1) has been extensively studied as a possible binding target. It is well known to be required for trans-endothelial migration (Zemans *et al*., 2009) and has been shown to be upregulated during inflammation in a variety of epithelial cells including alveolar, bronchial and tracheal (Burns *et al*., 1994, Look *et al*., 1992, Tosi *et al*., 1992). However, ICAM-1 is only found on the apical surface of epithelial cells and is therefore only in a position to act as a binding partner following TEpM when the cells are anchored to the epithelial surface, thereby explaining the failure of ICAM-1 antibody to block a rise in leucocyte counts following a stimulus (Zemans *et al*., 2009). Members of the JAM family provide an alternative ligand on

epithelial cells. Both JAM-A and JAM-C are ligands to β2 integrins but only JAM-C has been shown to be required for TEpM through its interaction with CD11b/CD18 (Zen *et al*., 2004).

Once the leucocyte is firmly adhered to the surface of the epithelial cell it must then pass across the epithelial barrier. Unlike in the endothelium this process cannot occur in a trans-cellular fashion but instead is purely via a para-cellular route (Zemans *et al*., 2009). In the lungs this happens preferentially at tri-cellular junctions between two type I alveolar cells and one type II alveolar cell as the adhesional complex is already disrupted at these sites (Burns *et al*., 2003). CD47 plays a key role in this paracellular migration as pre-incubation with blocking antibodies leads to a build-up of leucocytes at the apical surface as although they are able to adhere to the cells they do not move further (Parkos *et al*., 1996). CD47 appears to control this function through both its activation of tyrosine kinases and its interaction with Signal regulatory protein alpha (SIRPα) This in turn activates the phosphatases Src homology region 2 domain-containing phosphatase-1 & 2 (SHP-1&2) which are postulated to alter the epithelial cell architecture (Liu *et al*., 2001, Liu *et al*., 2004a). As outlined previously CAR at the epithelial cell membrane can also dimerise with JAM-L on leucocytes, and a loss or inhibition of this results in a loss of TEpM (Zen *et al*., 2005, Verdino and Wilson, 2011, Witherden *et al*., 2010).

Finally once the leucocyte has crossed the epithelium it can adhere to the epithelial surface despite physical factors such as coughing or pulmonary oedema. In so doing the leucocytes are able to remain in place to deal with the original inflammatory stimulus. This occurs through multiple binding partners including ICAM-1 with CD11B/CD18 and the Fc receptors on the leucocyte

binding to antibodies which are in turn bound to the ligands on the epithelial cell surface (Huang *et al*., 1996, Halstensen *et al*., 1990).

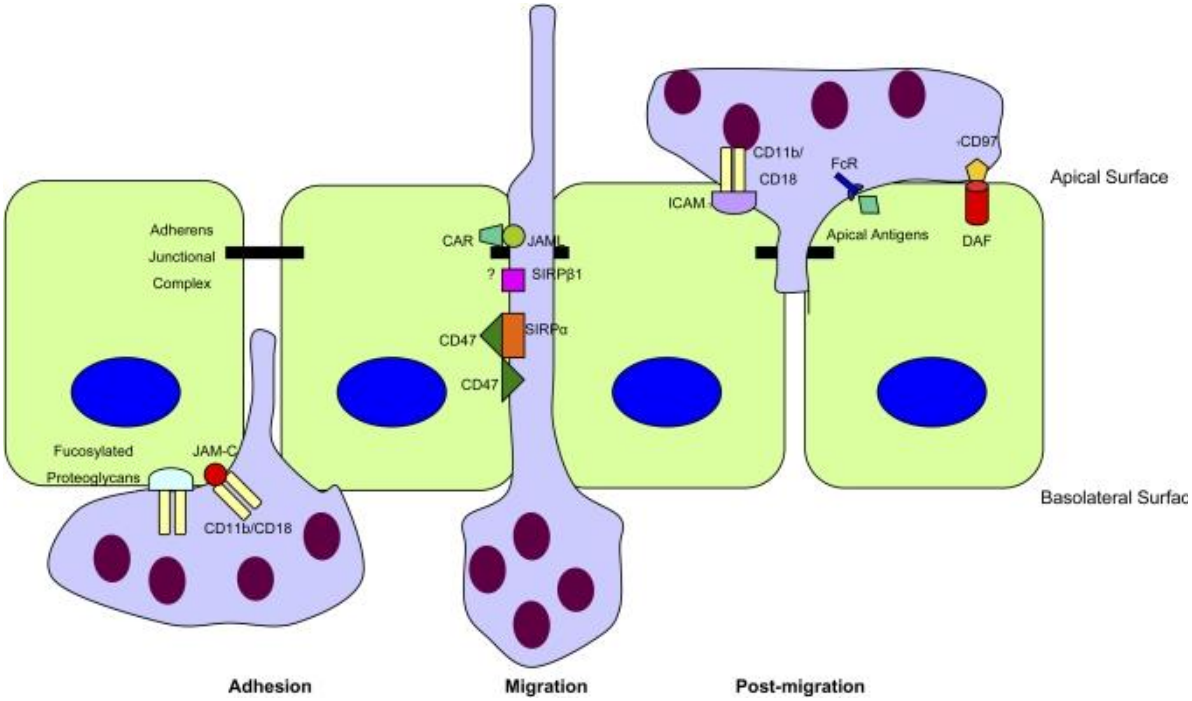


Figure 1-8: Leucocyte migration across an epithelial barrier into the lumen. TEpM occurs in 4 steps: 1) Leucocytes adhere to the basolateral side of the epithelium. 2) Trans-epithelial migration occurs along the basolateral membrane through interaction with adhesion molecules such as JAMs and CD47. 3) Leucocytes reach the apical side of the epithelium and are retained at the surface of epithelial cells by ICAM-1–Mac-1 and Ig–FcR (Fc receptor) interactions. 4) Finally neutrophils can get access to pathogens and to mediate an effective immune response. (Garrido-Urbani *et al*., 2008, Zemans *et al*., 2009).

A summary of the TEpM process is shown in figure 1.8 highlighting the three steps of adhesion, migration and post-migrational adhesion that happen. As described this is a tightly controlled process that ensures the epithelial barrier is maintained to prevent the movement of unwanted substances during the leucocytes passage. This includes the release of adenosine by transmigrating leucocytes to aid the reformation of tight junctions (Zemans *et al*., 2009, Lawrence *et al*., 2002). In pathogenic states, where there is a significant movement of cells, TEpM and the influx of leucocytes can lead to damage to the epithelial barrier. This can occur in several ways including direct physical damage

due to mechanical force resulting in epithelial wounds as well through the sustained release of inflammatory mediators such as cytokines by the leucocytes.

1.8 Cytokine driven cell signalling

The impact of cytokines on cell function is wide-ranging and reflects their ability to alter multiple cellular processes. One way of particular relevance by which they control the inflammatory response is through their effect on tight junctions. Interferon (INF) γ was the first cytokine shown to drive inflammation through its impact on tight junctions by causing an increase in tight junction permeability (Madara and Stafford, 1989). Since then multiple other cytokines have been implicated in both junctional disruption and stability including TNF α, IL-1β, IL-13 and IL10 (Al-Sadi *et al*., 2009). In particular in the intestine it has been shown that both in-vitro and *in vivo* cytokine derived barrier disruption of the epithelium leads to inflammation (Al-Sadi *et al*., 2009). Importantly this cytokine driven junction disruption can be blocked *in vivo* with the resultant loss of inflammation demonstrating a direct causal link for junctional disruption leading to inflammation (Schwarz *et al*., 2007). These findings though are not specific to the intestinal epithelium. For example in the lungs of asthmatic patients IL4 and IL-13 have also been indicated in causing epithelial disruption suggesting they play a role in propagating the chronic inflammation seen (Ahdieh *et al*., 2001). TNF α is another cytokine that has been shown to affect the tight junctions of a wide variety of cell types including the epithelial cells of the kidney, gut and lung as well as endothelial cells from the lung (Al-Sadi *et al*., 2009, Mullin *et al*., 1992, Marano *et al*., 1998, McKenzie and Ridley, 2007). Its effect on the junctions includes stimulating the internalisation of junctional proteins, JAM-A, occludin and claudins (McKenzie and Ridley, 2007, Ivanov *et al*., 2005) but TNF α exemplifies

the multiple pathways cytokines can activate to drive inflammation and therefore alternative pathways may also play a role in its impact on permeability.

1.8.1 Tumour necrosis factor alpha (TNF-α)

TNF is of specific interest given its established pleotropic role in the inflammatory response which can be both appropriate to ensure protection against infection (Waters *et al*., 2013) and also lead to a variety of unwanted effects at the epithelium in diseases such as in rheumatoid arthritis, psoriasis, asthma and inflammatory bowel disease (Baert and Rutgeerts, 1999, Berry *et al*., 2007, Holgate, 2010, Murdaca *et al*., 2009). This is of particular relevance in the respiratory epithelium as it has been shown in those patients with severe steroid resistant asthma that their airway hyper-responsiveness can be controlled through TNF α inhibition with etanercept (Holgate, 2010, Morjaria *et al*., 2008, Howarth *et al*., 2005, Berry *et al*., 2006). Although in a larger study the efficacy of anti-TNF α treatment was limited by its adverse effects profile (increased rates of infection and cancer), analysis did show that airway flow rates and inflammation did respond in a dose dependent fashion further supporting a role for TNF in inflammatory lung disease (Wenzel *et al*., 2009).

TNF α belongs to the large TNF superfamily which includes at least 19 different ligands. It is a pleotropic cytokine and as such acts upon almost all differentiated cells to trigger a wide range of biological responses including cell proliferation, differentiation and apoptosis along with lymphocyte and leucocyte activation and migration, as well as more systemic effects such as fevers and the acute phase response (Waters *et al*., 2013). TNF α is mainly produced by macrophages, monocytes and to a lesser extent B-cells, Natural Killer (NK) - cells, Kupffer and glial cells. It is either found as a membrane bound 34 KDa type II transmembrane

protein or following cleavage by the metalloproteinase enzyme, TNF α converting enzyme, to a 17 KDa soluble protein. Both these two forms of TNF α bind as a trimer to two distinct receptors, TNF receptor 1 and 2 (TNFR1 and TNFR2). These receptors have been shown to play a role in both infection and inflammation. Firstly they are required for successful *S. aureus* infection through their binding with protein A released by the bacteria (Gomez *et al*., 2004, Gomez *et al*., 2006). TNFR1 in particular has also been shown to play a role in the loss of intestinal epithelial barrier function that occurs with total parenteral nutrition through its upregulation (Feng and Teitelbaum, 2013). When TNF α binds to its receptors they undergo trimerisation to activate multiple different signalling proteins including TNF-receptor associated factor 2 (TRAF2), receptor interactin protein 1 (RIP1) and fas-associated death domain protein (FADD). TRAF2 activates the MAPK pathway, while RIP1 activates the nuclear factor kappa beta (NF-κB), which in turn translocates to the nucleus, binds DNA and induces or represses gene expression (Baud and Karin, 2001, Waters *et al*., 2013). Another process induced by TNF α is apoptosis and this is mainly mediated through FADD. NF-κB can inhibit apoptosis through induction of cellular inhibitors of apoptosis (cIAPs) (Baud and Karin, 2001). In Caco-2 epithelial cells TNF α induces activation of NF-κB pathways which lead to cytoplasmic-to-nuclear translocation of NF-κB, increased NF-κB binding to the DNA binding site, downregulation of ZO-1 protein expression, disturbance in junctional localisation of ZO-1 protein and functional opening of the TJ carrier (Ma *et al*., 2004).

Significantly TNF α has been shown to play a role in the stability and function of tight junctions in both endothelial and epithelial cells through its ability to control the function the proteins present in tight junction (Baert and Rutgeerts, 1999,

Fischer *et al*., 2013, Feng and Teitelbaum, 2013). Interestingly the presence of TNF α has also been shown to stimulate neutrophil infiltration of both endothelial and epithelial layers (Woodfin *et al*., 2009, Finsterbusch *et al*., 2014). Studies have furthermore indicated that the presence of TNF leads to the redistribution of JAM proteins in endothelial cells so that they are present at the apical surface of endothelial cells in order that they are in a position to facilitate leucocyte transmigration (Ozaki *et al*., 1999, Ostermann *et al*., 2002). Therefore TNF is altering tight junction composition in such a way as to ensure effective leucocyte movement. Pertinently for this study a TNF mediated increase in barrier permeability has been shown to be associated with a loss of epithelial junctional proteins including E-Cadherin and P-120 (Hardyman *et al*., 2013). This was also correlated with *in vivo* biopsy results that showed an equivalent loss of these junctional proteins and an increased number of neutrophils in the airways of asthmatic patients compared to healthy human subjects suggesting that it can play a role in epithelial junction disruption in disease and that this could lead to leucocyte TEpM in the lung (Hardyman *et al*., 2013).

Therefore TNF has been shown to affect both tight junction protein structure and function as well as drive leucocyte TEpM. Given the role CAR plays in tight junctions and leucocyte activity there is consequently the suggestion that the two are in a position to interact to control the inflammatory response through control of TEpM.

1.9 Aims and hypothesis of this study

CAR is a member of the tight junction complex and is known to act as a binding partner to facilitate the TEpM of leucocytes. However, it is currently unknown as to whether CAR plays active or passive role in this process and if so how this

might occur. We hypothesise that epithelially-expressed CAR is responsive to inflammatory cytokines, potentially through phosphorylation of CAR, and this controls the rate of passage of leucocytes across the lung epithelium.

In order to test this hypothesis, the aims of this study are to:

1. Determine whether sites on the cytoplasmic tail of CAR are phosphorylated in response to cytokine stimulus.
2. Identify possible signalling pathways that may control any phosphorylation effect.
3. Determine whether the phosphorylation of the cytoplasmic tail of CAR alters its interaction with leucocytes facilitating their transmigration.

2 Methods and Materials

2.1 Cell Lines

Human bronchial epithelial cells (HBEC) were originally from a 65 year old female with no evidence of cancer. They were immortalised by over expression of the cell cycle protein cdk4 which abrogates the p16/Rb cell cycle check point pathway and the catalytic subunit of the telomerase enzyme (human telomerase reverse transcriptase, hTERT) to bypass replicative senescence (Sato *et al*., 2006). They were kindly donated to us by Dr Jerry Shay from the University of Texas, Southwestern Medical Centre.

The human acute monocytic leukaemia cell line (THP-1) cells were a gift from Prof Gareth Jones at King's College London. They were originally purchased from American Type culture Collection (Rockville, MD). They are derived from a one year old male with leukaemia but have no identified chromosomal abnormality and can if required be stimulated to form mature macrophages.

2.2 Cell culture

HBEC (Human Bronchial Epithelial Cells) were maintained in Keratinocyte-SFM (K-SFM) Medium with L-Glutamine (Invitrogen) supplemented with Human recombinant Epidermal Growth Factor (EGF 1-53), Bovine Pituitary Extract (BPE) penicillin/streptomycin (Invitrogen)). The media was supplied in 500ml bottles to which the supplied aliquots of EGF 1-53 and BPE were added along with 5mls of the penicillin solution.

Flasks and plates were coated with a sufficient volume to cover the whole surface of the plastic (for example 3mls for a T25 flask) with 10% type I bovine collagen (BD biosciences) for 1 hour prior to sub-culturing. Following 1 hour the collagen

coating was removed to be reused and the surface rinsed with Phosphate-Buffered Saline (PBS) to remove any excess.

Cells were passaged by washing with warm PBS before being incubated with Trypsin/EDTA 5mM (Invitrogen) at 37^{o}C until they detached (approximately 5 minutes depending on the confluency on the flask). Cells were re-suspended in warmed fresh growth media before centrifugation at 200G for 5 minutes. Cells were grown in 25cm^2 tissue culture flasks with a filter cap (Greiner Bio-One CELLSTAR®) at 37^{o}C in a humidified incubator with an atmosphere containing 5% CO_2. They were split at 75-80% confluence.

THP-1 cells were maintained in RPMI-1640 medium (GIBCO) supplemented with 10% (v/v) heat inactivated foetal bovine serum(FBS) and 0.05mM of β-mercapto-ethanol. The cells were kept in suspension culture in complete growth media in 75cm^2 or 175cm^2 tissue culture flasks with a filter cap (Greiner Bio-One CELLSTAR®) at 37^{o}C in a humidified incubator with an atmosphere containing 5% $CO_{2.}$ Cells were kept at 40-50% confluence and split at 80-90%.

2.3 Antibodies

Table 1. Table of antibodies used during the project. WB: Western Blot, IHC: immunohistochemistry. IP: Immunoprecipitation.

Antibody Name	Species	Source	Use	Optimal dilution	Concentration
Anti-CAR H300	Rabbit	Santa Cruz	WB/ IHC	1:100	200ug/ml
Anti-CAR (clone RmcB)	Mouse	Milipore	WB/ IHC	1:500	1mg/ml

p-thr290/ser293CAR polyclonal antibody	Rabbit	Perbioscience (Thermofisher)	WB IHC	1:250 1:50	
p-tyr263CAR polyclonal antibody	Rabbit	Perbioscience (Thermofisher)	WB	1:250	
Anti-E-Cadherin [HECD-1]	Mouse	Abcam	IHC	1:100	Data not provided by supplier
Anti-E-Cadherin (MB2)	Mouse	Abcam	IHC	1:100	1mg/ml
TNFR1 antibody (MAB225)	Mouse	R&D	IHC Blocking	1:200 10µg/ml	1µg/ml
p38 MAPK	Rabbit	Cell Signalling	WB	1:1000	Data not provided by supplier
P-p38 MAPK (Thr180/Tyr 182)	Rabbit	Cell Signalling	WB	1:1000	Data not provided by supplier
p44/42MAPK (ERK1/2)	Rabbit	Cell signalling	WB	1:1000	Data not provided by supplier
P-p44/42MAPK	Rabbit	Cell signalling	WB	1:1000	Data not

(ERK1/2)(Thr202/Tyr 204)					provided by supplier
AKT (#9272)	Rabbit	Cell signalling	WB	1:1000	Data not provided by supplier
P-AKT (Ser473)	Rabbit	Cell signalling	WB	1:1000	Data not provided by supplier
GAPDH [6C5]	Mouse	GeneTex	WB	1:25000	12.8mg/ml
HSC-70	Mouse	Santa Cruz	WB	1:3000	200µg/ml
NF-Kb p65 (D14E12)	Rabbit	Cell Signalling	IHC	1:400	20ng/ml
Phospho-PKCδ/θ (SER643/676)	Rabbit	Cell Signalling	WB	1:1000	10ng/ml
PKC δ (SC-937)	Rabbit	Santa Cruz	IHC	1:200	5ng/ml
Anti-CLMP	Rabbit	Atlas	WB	1:200	0.1mg/ml
Anti-AMICA1 (JAM-L)	Rabbit	Sigma	WB	1:500	0.2mg/ml
Anti-GFP	Rabbit	MBL	IP	3µL/sample	
anti-phospho-serine	Rabbit	Cell signalling	WB	1:1000	0.25mg/ml
anti-phospho-	Rabbit	Cell signalling	WB	1:1000	0.25mg/ml

threonine					
anti-phospho-tyrosine (p-100Tyr)	Mouse	Cell signalling	WB	1:1000	0.25mg/ml
Alexa Fluor® 568 goat anti-mouse IgG	Mouse	Invitrogen	IHC	1:400	1.4mg/ml
Alexa Fluor® 488 goat anti-mouse IgG	Mouse	Invitrogen	IHC	1:400	1.4mg/ml
Alexa Fluor® 568 goat anti-rabbit IgG	Rabbit	Invitrogen	IHC	1:400	1.4mg/ml
Goat anti-mouse IgG-HRP	Mouse	Santa Cruz	WB	1:2000	400µg/ml
Goat anti-rabbit IgG-HRP	Rabbit	Santa Cruz	WB	1:2000	400µg/ml
Alexa Fluor® 633 phalloidin		Invitrogen	IHC	1:100	Data not provided by supplier

2.4 Cytokines

TNF α was produced in yeast and supplied by Sigma (10ng/ml). Interferon γ was produced in E.coli and supplied by Sigma (used at 5ng/ml). Recombinant Human IL-13 (3.5ng/ml), IL-5 (2.5ng/ml), IL-1β (2.5ng/ml), IL-17 (3.125ng/ml) were all produced in E.coli and supplied by R&D systems.

2.5 Antibody production and ELISA assays

The polyclonal antibodies to the specific phosphorylation sites on the CAR cytoplasmic tail were produced by Perbioscience on behalf of Thermofisher. Two rabbits were inoculated against the peptide sequence Ac-RTS(pT)AR(pS)YIGSNH-C in order to generate an antibody against the serine/threonine site on the tail. Two further rabbits were inoculated against with the peptide sequence EEK(pY)EKEV-C in order to create the tyrosine site antibody.

The rabbits were then bled at days 28, 56 and 72 following inoculation. ELISA assays were then performed to determine whether antibodies were generated against the peptide sequence identified as indicating phosphorylation at each site. This technique was used as it would show whether an antibody had been generated capable of specific binding to the protein sequence of interest as only this would remain fixed to the protein during the washing steps.

The ELISA assays were performed with a capture antibody coated onto wells of a microplate. The peptide sequence for the phosphorylation sites were then added to the wells and allowed to bind to the capture antibody for 2 hours at room temperature. These wells were then aspirated and washed 4 times with a TBS wash buffer to remove any unbound protein. Either the rabbit sera from the inoculated animals or negative control rabbit sera was then added to each well and incubated at room temperature for one hour in order to allow any antibody to the specific phosphorylation site to bind. The sera were then aspirated away and the wells again washed three times with a TBS buffer to clear any unbound material away. Diluted HRP conjugate was then added to each well and the plate covered and incubated at room temperature for 30 minutes. The wells were then

again thoroughly aspirated to remove the solution from each well and the liquid discarded, followed by a further 4 washes. A chromogenic substrate could then be added to each well and the plate was allowed to develop in the dark for 30 minutes before a 0.16 M sulphuric acid stop solution was added to each well. The plate was then evaluated using an optical plate reader within 30 minutes to evaluate the titre values for each bleed in comparison to the control samples.

The tables below show the crude data of the different bleed days for the 4 animals.

Table 2: CAR p-ser293/thr290 antibody data

Animal Number	Date	Day	Titer	Titer Type	Description
PA5829	09/12/2012	0	50	CrudeSera	Treatment
PA5829	09/12/2012	0	50	CrudeSera	Control
PA5829	10/10/2012	28	12800	CrudeSera	Control
PA5829	10/10/2012	28	51200	CrudeSera	Treatment
PA5829	11/07/2012	56	51200	CrudeSera	Control
PA5829	11/07/2012	56	102400	CrudeSera	Treatment
PA5829	11/23/12	72	1600	CrudeSera	Control
PA5829	11/23/12	72	51200	CrudeSera	Treatment
PA5830	09/12/2012	0	50	CrudeSera	Treatment
PA5830	09/12/2012	0	50	CrudeSera	Control
PA5830	10/10/2012	28	6400	CrudeSera	Control
PA5830	10/10/2012	28	51200	CrudeSera	Treatment
PA5830	11/07/2012	56	12800	CrudeSera	Control
PA5830	11/07/2012	56	204800	CrudeSera	Treatment
PA5830	11/23/12	72	3200	CrudeSera	Control
PA5830	11/23/12	72	102400	CrudeSera	Treatment

Table 3: CAR p-tyr269 antibody data

Animal Number	Date	Day	Titer	Titer Type	Description
PA5831	09/12/2012	0	50	CrudeSera	Treatment
PA5831	09/12/2012	0	50	CrudeSera	Control
PA5831	10/10/2012	28	12800	CrudeSera	Treatment
PA5831	10/10/2012	28	25600	CrudeSera	Control
PA5831	11/07/2012	56	51200	CrudeSera	Treatment
PA5831	11/07/2012	56	51200	CrudeSera	Control
PA5831	11/23/12	72	6400	CrudeSera	Treatment
PA5831	11/23/12	72	6400	CrudeSera	Control
PA5832	09/12/2012	0	50	CrudeSera	Treatment
PA5832	09/12/2012	0	50	CrudeSera	Control
PA5832	10/10/2012	28	25600	CrudeSera	Control
PA5832	10/10/2012	28	25600	CrudeSera	Treatment
PA5832	11/07/2012	56	102400	CrudeSera	Control
PA5832	11/07/2012	56	102400	CrudeSera	Treatment
PA5832	11/23/12	72	12800	CrudeSera	Control
PA5832	11/23/12	72	25600	CrudeSera	Treatment

2.6 PKCδ silencing

In order to determine the role played by PKCδ in the phosphorylation of the cytoplasmic tail of CAR its synthesis was inhibited using a small interfering RNA. This siRNA binds to the messenger RNA responsible for the production of PKCδ causing its degradation and therefore limiting the protein synthesis.

To do this 10 µL of 5 µM PKCδ siRNA targeting the GGGACACUAUAUUCCAGAAtt sequence (Ambion s11099) was added to 90 µL Opti-MEM® and 5 µL of DharmaFECT reagent was added to 95 µL of Opti-

MEM® and left for 5 minutes at room temperature. The DharmaFECT was used as a lipid based reagent that aided the siRNA's passage across the cell membrane and into the cell.

The two were then combined together and left for 20 minutes at room temperature. Then a further 800 µL of Opti-MEM® was added to the mixture and replaced the media covering HBEC which had been grown to a 50% confluence in 6 well plates and then incubated at 37 ^{0}C in 5% CO2. The media covering the control cells was replaced with Opti-MEM®. The Opti-MEM® +/- siRNA was replaced with HBEC media after 6 hours and the cells were grown for a further 48 hours.

2.7 Immunoblotting

These experiments were performed to determine the activity of specific proteins in response to different stimuli. This was predominantly focused on determining whether the cytoplasmic tail was being phosphorylated in the presence of certain cytokines. To do so cells were stimulated and then lysed with the aim of preserving the phosphorylation state of any protein.

HBEC were seeded in 6 well plates and grown to confluency in normal growth media. One hour pre-treatment media was replaced with 2mls of warm (37 ^{o}C) serum free media (Opti-MEM®) before the addition of treatments as indicated in the results section. Samples were then lysed in 300ul hot sample buffer (at 95^{o}C) containing β-mercaptoethanol to break disulphide bonds and open protein structures. The sample buffer was composed of Tris Cl, (pH 6.8, 60mM) to act as a buffer, Glycerol, (25%) to increase the density of the solution to ensure it settled into each well for SDS-PAGE, SDS (25%) to disrupt covalent bonds thereby inactivating enzymes as well as providing a negative charge, along with

bromophenol blue to aid visualisation. Lysates were immediately scrapped from their plates and heated at 95^{o}C for 10 minutes before being subjected to SDS-PAGE.

The separation of different proteins by molecular weight and charge was performed via electrophoresis using the Laemmli SDS-PAGE method with a 10% polyacrylamide gel for all CAR protein experiments and 4% stacking gel. Between 10 and 20 µl of the samples were added to each well depending on the volume of cassette used for each experiment. Samples were initially run through the stacking gel at 100V for 30 minutes before increasing to 160V to run through the separating gel (approximately 90 minutes). The running buffer used was purchased from Thermofisher Scientific (Novex® Tris-Glycine SDS Running Buffer (10X)) and diluted 10 times in distilled water.

At the end of the run the gels were transferred to nitrocellulose membranes. In brief, the gel/membrane was sandwiched between two 1x Transfer Buffer pre-soaked Whatman filter papers and two 1x Transfer Buffer pre-soaked blotting pads. The gel/membrane blot module was run for 90minutes at 30 volts in 1x Transfer Buffer (Novex® Tris-Glycine Transfer Buffer). The outer buffer chamber was filled with deionised water to dissipate heat produced during the run.

Upon completion of the run, the membranes were blocked for 30 minutes at room temperature in 5% Bovine Serum Albumin (BSA) in Tris-Buffered Saline Tween-20 (TBS-T) (Sigma). Membranes were incubated with the appropriate concentration of primary antibodies (section 2.3) in 5mls of 5% BSA TBS-T overnight at 4oC on a roller.

The following day the immunoblots were washed 3 times for 5 minutes in TBS-T and incubated for 1 hour at room temperature with the appropriate concentrations

of either anti-mouse or anti-rabbit horseradish peroxidase-conjugated (HRP) (Santa-Cruz Biotechnologies) in 10mls of 5% BSA TBS-T on a rocker at room temperature. It is the HRP that provides the means of detection. After incubation the immunoblots were washed 3 times in TBS-T for 15 minutes per time and placed on tissue paper to remove any excess buffer.

Detection was carried out using ECL Western Blotting Detection Kit (Amersham), this relies upon the HRP enzyme to oxidise luminal. The detection kit contains two solutions, labelled 1 and 2, which were mixed in equal quantities and added to the nitrocellulose membranes for one minute before bloating off. Then, in the dark room using a Bio-Rad developer after a set period depending on the amount of protein detected the film was developed and the bands were visualised.

For re-probing, blots were stripped using Re-blot 10x stripping buffer (Chemicon). Bound antibodies from the nitrocellulose membranes can be removed without having an effect on the immobilised proteins. Blots were treated with 1x stripping buffer diluted in distilled water. 20ml of solution was added to the blot and incubated for 15 minutes by gently agitating at room temperature. Subsequently, the blots were twice washed with 5% milk TBS-T for 15 minutes each time at room temperature. Blots were then re-probed with primary antibodies at $4^{O}C$ overnight for total protein or other proteins.

2.8 Immunoprecipitation

The aim of immunoprecipitation is to determine whether a specific protein is present within a complex. In this case the protein of interest was phosphorylated CAR with the addition of GFP which was captured from the complex by binding to a GFP SPECIFIC antibody which was in turn stabilised through its binding to A/G agarose beads. The CAR protein could then be eluted from beads and

analysed by SDS-PAGE, followed by Western blot detection using commercial anti-phospho antibodies. This process was used in the initial trial experiments before CAR phosphorylation specific antibodies were developed.

HBEC and CARGFP-HBEC were cultured in normal growth media for 24 hours before the media was replaced with a serum free media (Opti-MEM®) for one hour. Treatments were added as indicated in the results figures and then were washed with ice-cold phosphate-buffered saline (PBS) with the samples on ice. Samples were then lysed in 200 µl of IP lysis buffer (pH7.4 50mM Tris, 150mM NaCl, 1mM EDTA, 1% Triton, 1% NP40, PI cocktail). The cell lysates were then scraped into 1.5ml Eppendorf tubes and left on ice for 10 minutes. Cell nuclei and debris were removed by centrifugation at 13,000 rpm for 10 minutes. The supernatants were then transferred to new tubes and used for immunoprecipitation or stored at -80^{o}C.

Lysates were incubated with 50µl of A/G agarose beads overnight at 5^{o}C on a rotating wheel. The agarose beads were pre-prepared by rinsing 50 µl of beads with PBS before adding 3µg GFP antibodies (table 1) and leaving on the rotating wheel overnight. The GFP antibody was used as the CAR constructs all contained a GFP tag ensuring that the only protein that bound to the beads would need to contain GFP. To act as a negative control an empty GFP tag was transiently transfected into wild type HBEC cells using FuGENE as a non-liposomal transfection reagent to deliver the GFP DNA to the cells.

The following day the samples were centrifuged at 13,000 rpm for 10 minutes and 50 µl of unbound sample was saved. The remaining samples were then washed with 50µl of IP lysis buffer 3 times to clear any unbound sample. 30 µl of hot sample buffer (as described in section 2.7) with 5 µl of β-mercaptoethanol

was then added to the samples to remove the remaining bound protein from the agarose beads. The samples were then placed at 95 ^{o}C for 10mins and separated using SDS-PAGE and immunoblotted for anti-phospho-serine, anti-phospho-threonine or anti-phospho-tyrosine as described in section 2.7.

2.9 Immunostaining and confocal microscopy

Immunostaining aims to identify a specific cell antigen of interest. This is done with a similar principle as used for the previous immunoblotting experiments by using the antibody binding to specific antigens. Using immunostaining and confocal microscopy additionally requires an antibody to be linked to a fluorophore. The first, primary antibody, binds to the antigen of interest and then a secondary antibody with a fluorophore binds to this. The fluorophore can then be excited by a certain wavelength of light which then emit a different wavelength that is detected. Figure 2.1 shows the principle behind the confocal microscope where the laser provides the energy to excite the florescent molecule using the dichromatic mirror to filter out any unwanted wavelengths of light. The quality and resolution of the image is then improved by the pin hole which blocks any extraneous light that does not come from the point of focus. The detector can then build up a three dimensional image by adding separate images together taken from individual slices through the sample.

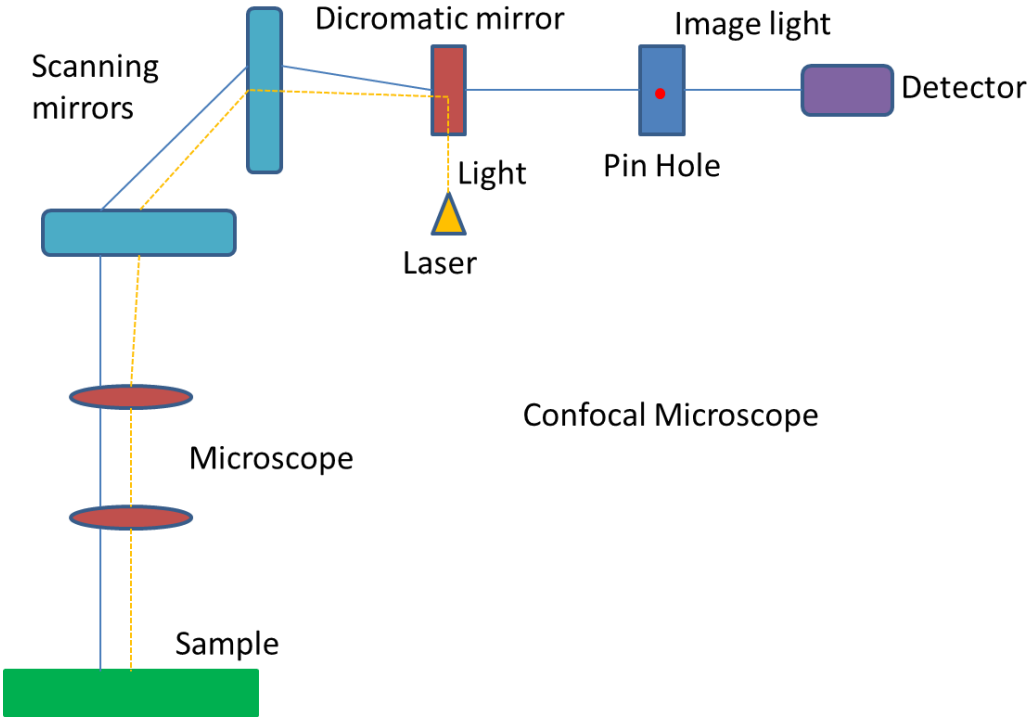


Figure 2-1 Graphic showing the principles of a confocal microscope.

Cells were cultured in 12 well plates on glass coverslips. They were then treated as described in the results section. Following treatment they were washed by briefly immersing them in PBS and fixed with 4% PFA in PBS for 10min and permeabilised with 0.2% TritonX-100 for 10min. Samples were blocked with a 5% BSA solution for 30 minutes and were then incubated with primary antibodies for 2 hours. They were then washed by again immersing in PBS followed by dH2O and the appropriate secondary antibodies conjugated to Alexafluor-568 or cy5 and Phalloidin conjugated to Alexafluor 568 or 633 where added in 5% BSA for 1 hour before once again washing in PBS followed by dH2O. Cells were mounted onto slides using Immunofluore (ICN) in order to protect the fluorescence of the secondary antibody. Confocal microscopy of HBEC alone was performed using an LSM510 Zeiss upright confocal microscope using a 63x oil objective or a Nikon A1R inverted confocal microscope using a 60x oil objective and laser excitation wavelengths of 405nm (for hoechst nuclear staining) 488nm (for GFP

or Alexafluor-488), 543nm (for Alexafluor-568) and 633nm (for Alexafluor-633 and cy5). Images were exported as tif files and prepared for figures using Photoshop.

2.10 TNFR1 inhibition

30,000 HBEC were seeded in collagen coated 6 well plates and grown to confluency in 2 millilitres of normal growth media. The wells had been coated in 10% type I bovine collagen for a minimum for one hour before the collagen was removed to be reused and the wells rinsed with PBS. The TNFR1 or control antibodies were added at a concentration of 10µg/ml for one hour prior to further treatment. HBEC cells were then further subjected to either the addition of 10ng/ml of TNF α or 100,000 THP-1 cells. The TNF α treated cells were then lysed after one hour as described in section 2.7. Where THP-1 cells were added the KSFM media was replaced with RPMI media. They were left for 4 hours before the media was removed and the cells lysed as described in section 2.7.

2.11 Permeability Assay

HBEC cells (either CARGFP or Wt populations) were seeded in 6.5mm Transwell chambers (Corning) with 8.0µm pores at 100,000 cells per well in 0.5 ml media in 12 well, collagen coated wells, with 1ml media in external part of the well (Figure 2.2). Wells were reviewed after 24hrs to ensure an even covering of cells with a stable complete monolayer. This step was fundamental to ensure an even monolayer of cells. Layers with obvious gaps between cells or where there was significant clumping of cells were disregarded as providing unrepresentative results. Equal numbers of CARGFP and Wt cell wells were required to ensure comparative data. 5µM TNF α was added to the specific treatment wells, followed by 10 µl of fitc-dextran (20 KDa) solution added to the upper chamber of all wells.

100 µl of the media was then collected from the lower chamber at 30 minutes and one hour after addition of fitc-dextran. Between collection points the samples were returned to the incubator to be maintained in their optimum conditions. The collected fluid samples were then compared for relative FITC-dextran concentration on a Fluostar Omega fluorescence plate reader (BMG). The system compared light absorption from each sample to provide a comparative reading of light transmission between the samples.

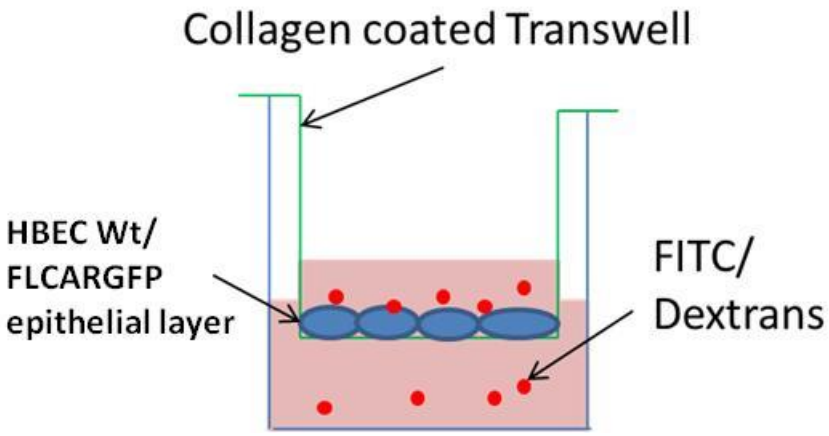


Figure 2-2: Permeability model showing cells grown on a collagen coated plate for a FITC/dextran permeability assay.

2.12 Transmigration Assay

HBEC cells were seeded in 6.5mm Transwell chambers (Corning) with 8.0µm pores at 30,000 cells per chamber and allowed to form monolayers. After 48 hours media in the upper and lower chambers was changed to RPMI containing 10% FBS before the addition of 100,000 THP-1 cells stained with Cell Tracker™ Orange Dye (Molecular probes) to the top well of the chambers. After 48 hours, the number of THP-1 cells in the lower chamber was counted using a FACSCalibur flow cytometer (BD Biosciences). For Ad5 fibre-knob competition assays recombinant Ad5FK (100µg of an 11mg/ml stock concentration) or BSA

control (100µg of 11mg/ml stock concentration) was added to HBEC cells 3 hours after seeding and remained in the media throughout the experiment.

2.13 THP-1 cell adhesion assay

HBEC cells were seeded onto 13mm coverslips and allowed to form monolayers. After 48 hours 100,000 THP-1 cells stained for 30 minutes with Cell Tracker^{TM} Orange (Molecular Probes) were added to the monolayers in RPMI supplemented with 10% FBS. THP-1 cells were allowed to adhere to HBEC monolayers for 16 hours before fixation and immunostaining with Phalloidin-633. A total of 5 5x5 tile-scans per sample were obtained using a 40x air objective on a Nikon A1R confocal microscope. The tiles were assembled together using Nikon NIS Elements software and further analysed using Cell Profiler (BD) to count the number of adhered THP-1 cells per image.

2.14 Organotypic culture

Organotypic cultures were performed as an alternative strategy to mimic physiological conditions whilst maintaining the use of immortalised cells with CAR alterations. Stromal cells are of fundamental importance in overall epithelial function and a more relevant assay therefore incorporates stromal components such as fibroblasts, and also reproduces the 3D characteristics of the relevant organ.

The cultures were established as shown in figure 2.3. Firstly a fibroblast containing gel was made using a bovine collagen type 1 (Millipore)/ matrigel (VWR international) mix (1:1 ratio) with 10x DMEM (Sigma), foetal calf serum, fibroblast cells (5 x10^5 cells per gel) and 7.5% sodium bicarbonate (at a ratio of 7:1:1:1). This is all performed on ice to prevent the matrigel from forming a gel before the mixture was complete. Sodium hydroxide was additionally added in a

drop wise fashion to neutralise the solution (the solution changes from yellow to pink). The gel mix was then placed in a 24 well plate and left to set at 37 ^{o}C for 1hour before 1ml of DMEM was added to the top and they were left over night. The following day HBEC cells (5 x 10^5) were added to the top and they were left to settle overnight. The nylon sheets shown in figure 2.3 were soaked in the same gel mixture as the original gel mixture aside from the lack of matrigel and fibroblasts. The sheets were then fixed using 1% glutaraldehyde. The nylon sheets and gels were placed on the steel grids as shown in figure 2.1. The media was replaced on alternate days for 10-14 days to ensure stable epithelial layer development. The gel/epithelial layer mixture was then submerged in 4% paraformaldehyde to fix them. Paraffin embedded sections were then prepared of the gels by the histology department at St Thomas' Hospital and slides stained and viewed via confocal microscopy as described in section 2.9.

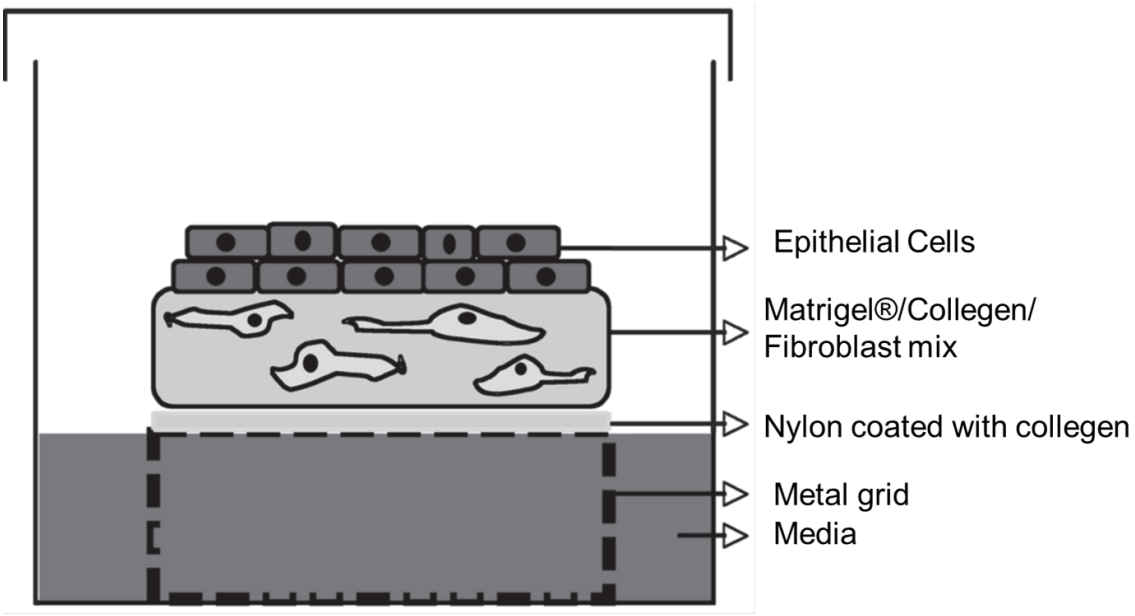


Figure 2-3: Organotypic culture showing HBEC cells grown at an air liquid interface on a matrigel/collagen/ fibroblast layer

No data from these experiments has been included in the thesis from these experiments. This is due to the technical and financial constraints these

experiments entailed. The technique is a slow process requiring at least one month for the culture and fixation of the experiment. During this process the samples are prone to infection as they cannot be grown in standard antibiotic media as used with most cell culture. The samples processed were also fragile and failed to establish stable three dimensional structures which reduced their relevance to a study of tight junction proteins. Mouse models were also developed in synchrony with these experiments and given their success were focused on.

2.15 Mouse models

2.15.1 Acute inflammatory mouse lung model

C57BL/6 (B6) mice (Harlan) were used at 4-8 weeks. All experiments were approved by our Institutional Animal Welfare Committee under UK Home Office Regulations. For mucosal sensitization 1µg recombinant murine TNF α (Immunotools) was given intranasally in 50µl PBS/mouse under light inhaled anaesthesia (isoflurane). After 24 hours animals were killed and bronchoalveolar lavage (BAL) performed using 1ml PBS.

Flow cytometry was used to identify the separate immune cell types present in the BAL fluid. This technique is useful as it allows large numbers of cells to be separated by type rapidly. This is based on the principle that cells passing through a laser beam will scatter light, which is detected as forward scatter (FS) and side scatter (SS). The combination of scattered and fluorescent light is detected and analysed. If the cells are pre-treated with antibodies that are known to bind to certain cell types the degree of forward and side scatter of light can be predicted and gated when stimulated by specific wave lengths of light.

Inflammatory cells in the BAL samples were identified as described (van Rijt *et al*., 2004). This used auto fluorescence of macrophages, and simultaneous one-step staining with antibodies for T cells (CD3-Cy-Chrome), B cells (B220-Cy-Chrome), eosinophils (CCR3-PE), and dendritic cells (DCs) (MHCII-FITC, CD11c-APC) with the addition of anti-Gr-1 to identify neutrophils ($Gr\text{-}1^{+}CD11c^{-}CCR3^{-}CD4^{-}CD8^{-}B220^{-}$). Antibody staining (0.1µg/sample, all eBioscience) was performed in PBS 1% FBS with each BAL sample after washing and was analysed on a FACScalibur (BD Bioscience). Total cell numbers were calculated by analysis of fixed sample volumes, validated with fluorescent beads. Figure 2.4 shows an example of the gating strategy used to identify the separate cell types.

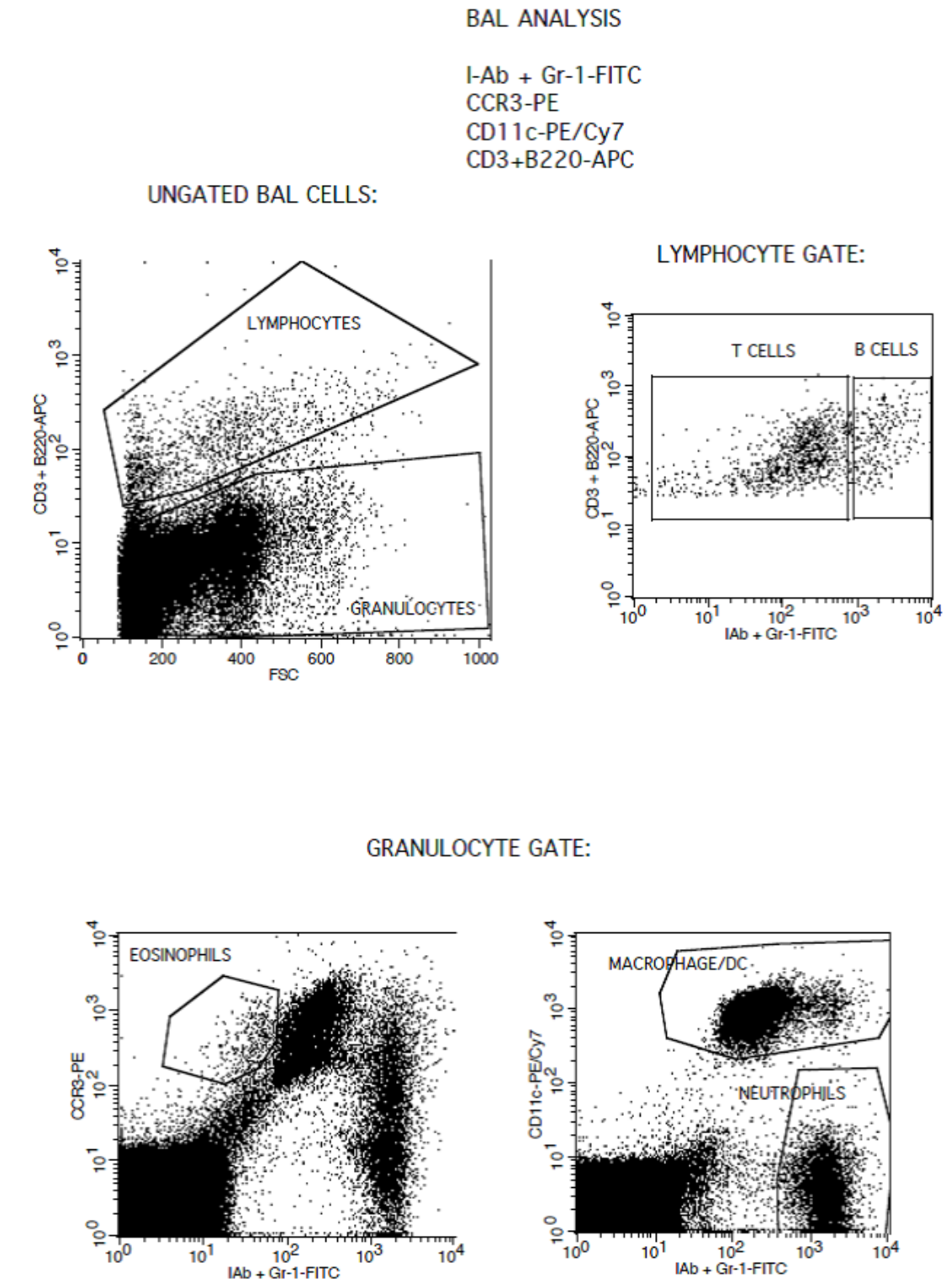


Figure 2-4: FACS gating strategy for BAL cell data.

2.15.2 Chronic inflammatory mouse lung model

Paraffin embedded slides of the mouse ovalbumin lung model sections were kindly supplied by Dr Gisli Jenkins, University of Nottingham. These slides were generated from BALB/c 6-wk-old mice, sensitized by i.p. injection of 10 µg OVA diluted 1:1 with adjuvant, followed by a second sensitization on day 12. At day 19, mice were challenged daily by oropharyngeal administration of either 400 µg/ml OVA in 50µl saline or 50 µl saline alone for 6 d, followed by additional challenges on days 26, 28, 30, and 33. The mice were sacrificed on day 34. For formalin-fixed tissue, the left lobe was inflated with formalin and fixed in formalin overnight and then embedded in paraffin wax. The slides were initially heated at 75 ^{o}C to warm the paraffin. The slides were then rehydrated via a step wise process of 2x immersion in xylene (supplied by Fisher scientific) for 10 minutes followed by 2 x immersion in 100% ethanol, 1x immersion in 90% ethanol, 1x immersion in 70% ethanol, 1 immersion in 50% ethanol all for 5 minutes each. Antigen retrieval was then performed in a citrate buffer at a pH of 6 in a pressure cooker for 15minutes. Slides were then washed in TBS and TBS tween and antibody staining was undertaken with the method as described in section 2.9.

3 Characterising the effects of cytokines on CAR phosphorylation and function

3.1 Introduction

3.1.1 Cytokines and Tight Junction Proteins

Cytokines are a broad group of small proteins that are known to both alter the constituents and function of cell junctions (Al-Sadi *et al*., 2009) and activate protein kinases responsible for serine, threonine and tyrosine phosphorylation (Thomas and Brugge, 1997). Interferon γ was the original cytokine implicated in tight junction control and function (Madara and Stafford, 1989) and is a pro-inflammatory cytokine principally secreted by lymphocytes in order to activate macrophages and drive a THP-1 immune response (Schroder *et al*., 2004). As part of this pro-inflammatory response it has also been shown to increase trans-epithelial permeability through disruption of tight junctions by increasing for example the micropinocytosis of occludin, JAM-A and claudin-1 (Madara and Stafford, 1989, Bruewer *et al*., 2005, Utech *et al*., 2005, Boivin *et al*., 2009, McKay *et al*., 2007, Ivanov *et al*., 2005). TNF α has also been shown to effect the function of tight junction proteins. TNF α exposure has been associated with disruption of tight junctions measured by a loss of trans-epithelial resistance (TER) (Mullin *et al*., 1992, Ma *et al*., 2004, Fish *et al*., 1999). Interestingly TNF α has also been associated with a reduction of tight junction complexity and activation of both protein kinases A and C as well those tyrosine kinases inhibited by genistein (Schmitz *et al*., 1999, Coyne *et al*., 2002). By affecting these kinases there are implications for any phosphorylation events occurring at associated tight junction proteins, as for example occludin, E-cadherin and β-catenin have all been shown to have their place in cell junctions affected by their phosphorylation

status (McCole, 2013). Significantly, VE-cadherin specifically has been shown to undergo tyrosine phosphorylation in response to the presence of TNF α (Angelini *et al*., 2006). Similarly IL-1β has also been implicated in the permeability of epithelial layers by increasing Na^+ and Cl^- passage through respiratory epithelium which is a process controlled by tight junction proteins (Coyne *et al*., 2002). IL-13 is another cytokine associated with tight junction disruption (Sanders *et al*., 1995) and although this has not been demonstrated in the respiratory epithelium interestingly it has been linked to activation of P1-3K (Prasad *et al*., 2005) which has also been shown to be activated by TNF α as previously stated (Kilpatrick *et al*., 2002). IL-17 is an additional cytokine linked with loss of tight junction integrity via the associated activation of ERK (Kinugasa *et al*., 2000).

A wide range of cytokines have also been studied and shown to have a deleterious effect in relation to tight junction function including IL2, 4, 6, 10 and 15. However, IL-3, 5, 7, 8, 9 12, 14, 16 have not been found to have an effect on tight junction stability suggesting that disruption of cell junction integrity is not a general function of pro-inflammatory cytokines (Al-Sadi *et al*., 2009).

Given our previous findings that the cytoplasmic tail of CAR can be phosphorylated (Morton *et al*., 2013), and work by others showing that cytokines can cause functional changes in tight junction proteins by their phosphorylation (Angelini *et al*., 2006), along with the important role of CAR in immune cell migration (Verdino *et al*., 2010, Witherden *et al*., 2010, Zen *et al*., 2005), we sought to investigate whether CAR might be phosphorylated in response to cytokine driven inflammatory conditions.

3.1.2 Objectives

The aim of experiments described in this chapter is to characterise the effect of cytokine stimulation on the putative phosphorylation sites on the cytoplasmic tail of CAR. Data demonstrates that the presence of inflammatory cytokines lead to phosphorylation of the cytoplasmic tail of CAR and the sites where this occurs. Moreover, further analysis shows TNF α to be the cytokine most strongly stimulating phosphorylation of CAR and this occurs through defined signalling pathways. Finally these data suggest that CAR recruitment, homodimerisation and maintenance at cell-cell adhesions is necessary for this TNF α-phosphorylation response to occur.

3.2 Results

3.2.1 Phosphorylation of CAR in response to cytokines

Our group has previously identified via *in silico* analysis two possible sites for PKC phosphorylation within the cytoplasmic tail of CAR; threonine at the amino acid 290 and serine at amino acid 293 (see Figure 1.7 in the introduction). To investigate these sites further, the group have previously established HBEC cells that either overexpress CAR (CAR-GFP) or alternative CAR cytoplasmic tail mutants that would either mimic phosphorylation by replacing the serine and threonine amino acids with aspartic acid (DDCAR-GFP) or would be incapable of being phosphorylated by replacing them with alanine (AACAR-GFP) (Morton *et al*., 2013). Using these cell lines we have previously shown that CAR is serine/threonine phosphorylated (Morton *et al*., 2013). This phosphorylation response altered the junctional protein components by controlling E-cadherin dynamics at the cell membrane thereby establishing a functional role for phosphorylation of the cytoplasmic tail of CAR.

Our group has additionally identified a potential Src kinase phosphorylation motif on the cytoplasmic tail of CAR; Tyrosine 269. HBEC cell lines were generated using CAR-GFP mutants that either block phosphorylation of this site by replacing the tyrosine residue with the similar, but non-phosphorylateable phenylalanine (Y2F CAR-GFP), or mimic phosphorylation by replacing the tyrosine with glutamic acid (Y2E CAR-GFP) at this site. Figure 3.1 highlights the difficulty inherent with this process as the tyrosine phospho-mimic substitution; glutamic acid is significantly structurally different to phosphotyrosine.

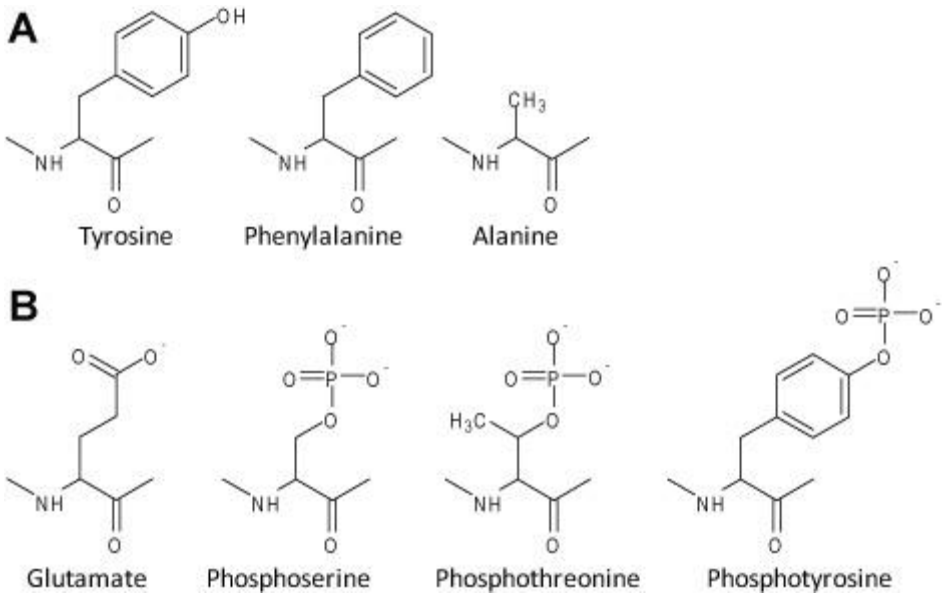


Figure 3-1 Alternative amino acids for tyrosine substitution.
A) Comparison of tyrosine with the alternative phospho-dead amino acids. B) Comparison of the phospho-mimic amino acid alternative glutamate with phosphoserine, phosphothreonine and phosphotyrosine.

To examine the role of CAR in inflammatory processes we first analysed the effect of pro-inflammatory cytokines on the phosphorylation status of CAR using CAR-GFP HBECs. TNF α and interferon γ were chosen for an initial trial of CAR's response to inflammatory conditions as they have long been established as playing a key role in the function of tight junctions in the epithelial response to inflammation both individually and in combination (Al-Sadi *et al*., 2009, Madara and Stafford, 1989, Mullin *et al*., 1992, Fish *et al*., 1999). The cells were alternatively treated with protein phosphatase inhibitors for serine/threonine sites (calyculin A) and tyrosine sites (pervanadate) as positive controls to the possible phosphorylation sites identified on the CAR cytoplasmic tail. Wild type (Wt) HBEC were also transiently transfected with an empty GFP tag (eGFP) to act as a negative control. These samples were then immunoprecipitated using an anti-GFP antibody and subjected to SDS-PAGE and western blot using pan phospho-serine, phospho-threonine or phospho-tyrosine antibodies (Figure 3.2). This

revealed that CAR serine and threonine phosphorylation was induced after TNF α but not interferon γ treatment. Neither cytokine treatment induced a detectable phospho-tyrosine signal despite a strong pervanadate induced signal (figure 3.2).

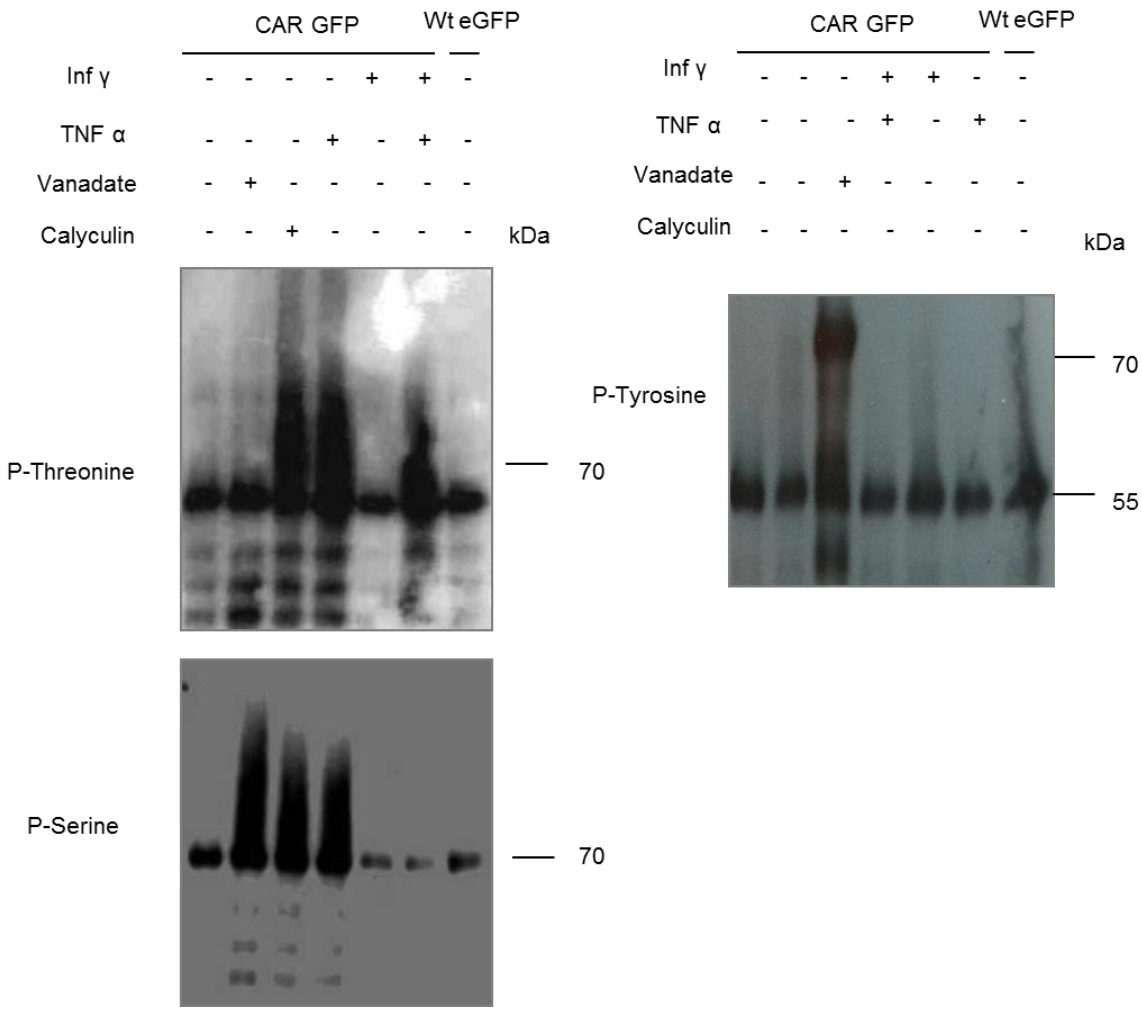


Figure 3-2 The cytoplasmic tail of CAR is phosphorylated at both the threonine and serine sites by TNF α but not at the tyrosine site or by Interferon γ. Western blot of immunoprecipitated CAR-GFP from HBEC lysates treated as indicated with either 30 µM Pervanadate, 0.5 mM Calyculin A, 10ng/ml TNF α or 5ng/ml Interferon γ as indicated. Western blots were probed with pan phospho-tyrosine, -serine or –threonine antibodies (Sampling repeated 4 times per test condition). The bands at 70 kDa are appropriate for a phosphorylation response by CAR. A response is seen to calyculin, TNF α and TNF α/INFγ with P-threonine, Vanadate, calyculin and TNF α with P-serine but only vanadate with P-tyrosine.

3.2.2 Development of serine/threonine phosphorylation specific CAR antibodies

Using pan-serine and threonine antibodies allowed identification of TNF α mediated CAR ser/thr phosphorylation via immunoprecipitation. However, this method does not allow us to specifically target the identified sites on the CAR cytoplasmic tail. We therefore developed polyclonal antibodies that were raised against the target sequence on the cytoplasmic tail containing the serine and threonine sites (peptide sequence Ac-RTS(pT)AR(pS)YIGSNH-C). Given the proximity between the 290 serine site and the 293 threonine site it was not possible to develop antibodies to recognise each one individually. The peptide was designed therefore to encompass both. The use of a polyclonal antibody as opposed to a monoclonal antibody did reduce cost and significantly allowed for greater tolerance of minor changes in the antigen. However, it does lead to limitations with an increased chance of non-specific signals and if further antibody was required would risk problems from inter batch variability.

Two separate rabbits were inoculated with the peptide and then bled on days 28, 56 and 72. The sera from these bleed days was used to probe for CAR phosphorylation of the serine and threonine sites in western blots of CARGFP-HBEC cells. These cells were either untreated, treated with a serine and threonine phosphatase inhibitor (Calyculin A) as a positive control or pervanadate as a negative control. The strongest western blot response using the sera occurred from the day 72 bleed in rabbit PA5829 and day 28 for rabbit PA5830 as they had the most sensitive and specific response to CAR phosphorylation (figure 3.3a). The CAR phosphorylation effect in these cell lysates was confirmed using a commercial GFP antibody (figure 3.3b) which has previously been used

to indicate CAR phosphorylation with the presence of a double band in response to calyculin (Morton *et al*., 2013). Figure *3*.3c shows ELISA data performed by PERBIOSCIENCE (THERMOFISHER) antibodies using the phosphorylation antigen. Data shown demonstrate a strong titre response to the sera taken on day 72 for rabbit PA5829 and on day 28 from rabbit PA5830.

Serum from rabbit PA5829 was further purified following a terminal bleed to produce the final CAR p-ser293/thr290 antibody. This antibody was used to probe for CAR phosphorylation in western blot of CAR-GFP HBEC lysates. These cells were again either untreated or treated with the phosphatase inhibitors Calyculin A (pan-serine/threonine phosphatase inhibitor) or vanadate (pan-tyrosine phosphatase inhibitor). Western blots using this purified polyclonal antibody showed the expected band at approximately 70 KDa corresponding to phosphorylated CAR-GFP, but also bands at 55KDa (figure 3.4a). Figure 3.4b shows the same lysates probed using a commercial antibody to CAR (CAR H300 antibody, Santa Cruz Biotechnology). No additional band was seen at 55KDa indicating that the antibody was not detecting a specific cleaved portion of CAR. The experiment was repeated with WT HBEC, which express CAR at very low levels, and AACAR-GFP HBEC, which overexpress mutated CAR that had been modified to prevent phosphorylation at the serine/threonine site (figure 3.4c). In this case only the CAR-GFP cells have the expected band at approximately70 KDa in response to calyculin, indicating the phosphorylated protein. The 55KDa band, however, is present in all 3 cell lines suggesting that this is not specific to CAR. Given the extensive similarity with CAR like membrane protein (CLMP) and the fact that this protein has a predicted molecular weight of 55KDa the samples were also probed using a commercially available antibody against CLMP to determine whether the antibody was detecting this protein instead. The antibody

does pick up the presence of a band at 54KDa, which would indicate the presence of CLMP in these cell lysates. Yet the additional strong band with the phospho-specific antibody is several KDa higher and therefore it does not likely represent binding to CLMP (figure 3.4d).

The CAR p-ser/thr antibody was then used to analyse CAR phosphorylation by confocal microscopy. Phosphorylated CAR was identified specifically, as expected, at the cell-cell contacts of CARGFP HBEC and not in either WT or AACARGFP (phosphodead) HBEC (Figure 3.4e).

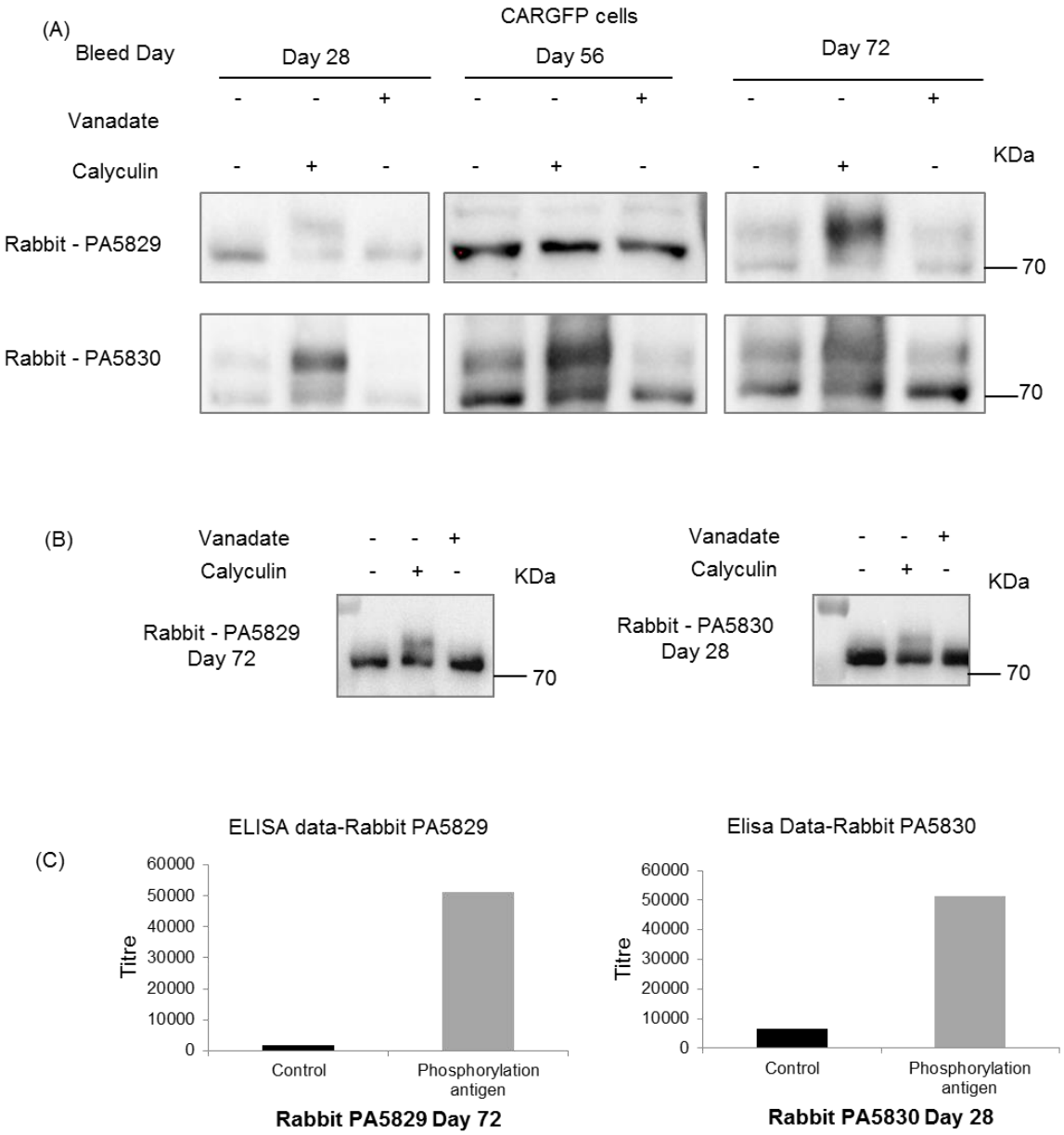


Figure 3-3 Phospho-Serine/threonine antibody development.
A) Western of HBEC lysates using the initial antibody developed from the donor rabbits on the specified bleed days. The bands seen at 72 kDa represent the serine/threonine site when it is phosphorylated on CAR-GFP cells (N=3). B) Western of the same lysates probed using the commercial antibody to GFP showing the double band associated with CAR phosphorylation (n=3). C) ELISA data for antibody titers from rabbits PA5829 and PA5830 on the specified bleed days to correspond to strongest and cleanest western data shown in image 3.3a.

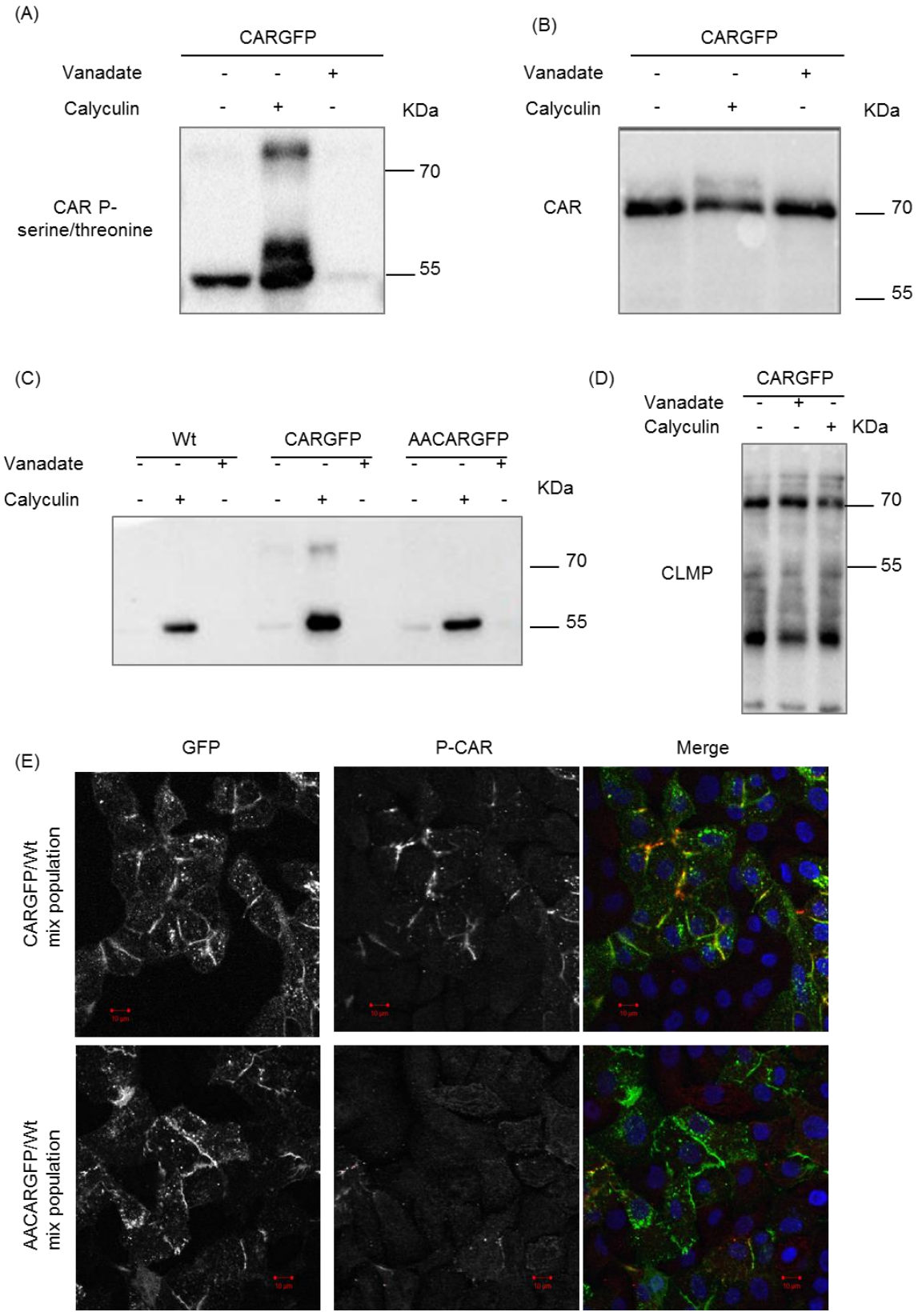


Figure 3-4 Serine/threonine CAR phosphorylation antibody purification.
A) Western of CAR-GFP lysates treated as indicated with 30 µM Pervanadate or 0.5 mM Calyculin A probed with purified CAR P-ser/thr antibody with the appropriate band seen at approximately 72 kDa as well additional bands at

55kDa. (n=3) B) Western of the same CAR-GFP lysates probed with the H300 CAR Santa Cruz antibody with no additional 55kDa band (n=2). C) Western of Wt HBEC, CAR-GFP HBEC and AACAR-GFP HBEC lysates treated as previously with band seen in CAR-GFP cell line following treatment with serine/threonine phosphatase inhibitor at 72kDa but not in the other cell lines. All three cell lines show an equivalent band at 55kDa (n=3). D) Western of CAR-GFP lysates probed for CAR Like Membrane Protein. Band seen at approximately 54kDa therefore below the non-specific band seen with the serine/threonine phospho-specific antibody (n=1). E) Confocal images using the serine/threonine phospho-specific antibody on CAR-GFP and AACAR-GFP cells treated with PDBu to drive phosphorylation. Presence of both CARGFP and phosphorylated CARGFP indicated by the yellow staining (n=3).

3.2.3 CAR phosphorylation at the serine/threonine phosphorylation site in response to TNF α

The development of a serine/threonine CAR phosphorylation site specific antibody allowed for further investigation of the sites response to cytokine stimulation. Initial data showed that CAR was ser/thr phosphorylated in response to TNF α stimulation in CAR-GFP HBEC cells. To confirm that TNF α induces phosphorylation of CAR at thr290/ser293 specifically, CAR-GFP was immunoprecipitated from untreated or TNF α treated HBEC cell lysates (figure 3.5a). TNF induced rapid phosphorylation of CAR at thr290/ser293 within 15 minutes, which diminished by 1 hour after treatment. Western blot of CAR-GFP HBEC lysates revealed similar induction of CAR phosphorylation at thr290/ser293 however with different kinetics (figure 3.5b). This may be due to differences in cell culture methods prior to treatment of the HBEC. CAR-GFP HBEC were treated and lysed when semi-confluent for immunoprecipitation (24 hours post-seeding) and when fully confluent (72hours post-seeding) for western blots shown in figure 3.5b. This effect was seen with multiple repeats of both short and longer-term cell culture. Quantification of western blots from multiple experiments confirmed the finding that there was a delay in the phosphorylation with increasing cell density and time in culture (figures 3.5c and 3.5d). This variation possibly reflects the difficulty for TNF α in accessing the TNF receptor with increasing cell density and increasing maturity of cell junctions following a longer culture period. This phosphorylation effect on the cytoplasmic tail of CAR in response to TNF α occurred at the cell membrane suggesting that CAR may be phosphorylated only when localised to cell junctions and may also need to be homodimerised for phosphorylation to occur (figure 3.6).

The over expressing CAR-GFP cell line showed increased CAR phosphorylation at the cell membrane following TNF α treatment (figure 3.5a) whereas the control AACAR-GFP cell line which are unable to be phosphorylated at the serine/threonine site showed no phosphorylation (figure 3.5a). Phosphorylated CAR has been found in internalising vesicles during cell junction disassociation caused by calcium washout (Morton *et al*., 2013). These p-se290r/thr293 CAR positive vesicles were not seen following TNF treatment (figure3.6), which could either be due rapid de-phosphorylation following internalisation or that the junctions themselves had not yet started to disassociate, as the previous study showed that cells with stable junctions did not have p-ser290/thr293 CAR positive vesicles present. Although this is only one time point this also suggests that the internal pool of CAR found in cells is not phosphorylated and instead needs to be trafficked to the cell membrane before this can occur.

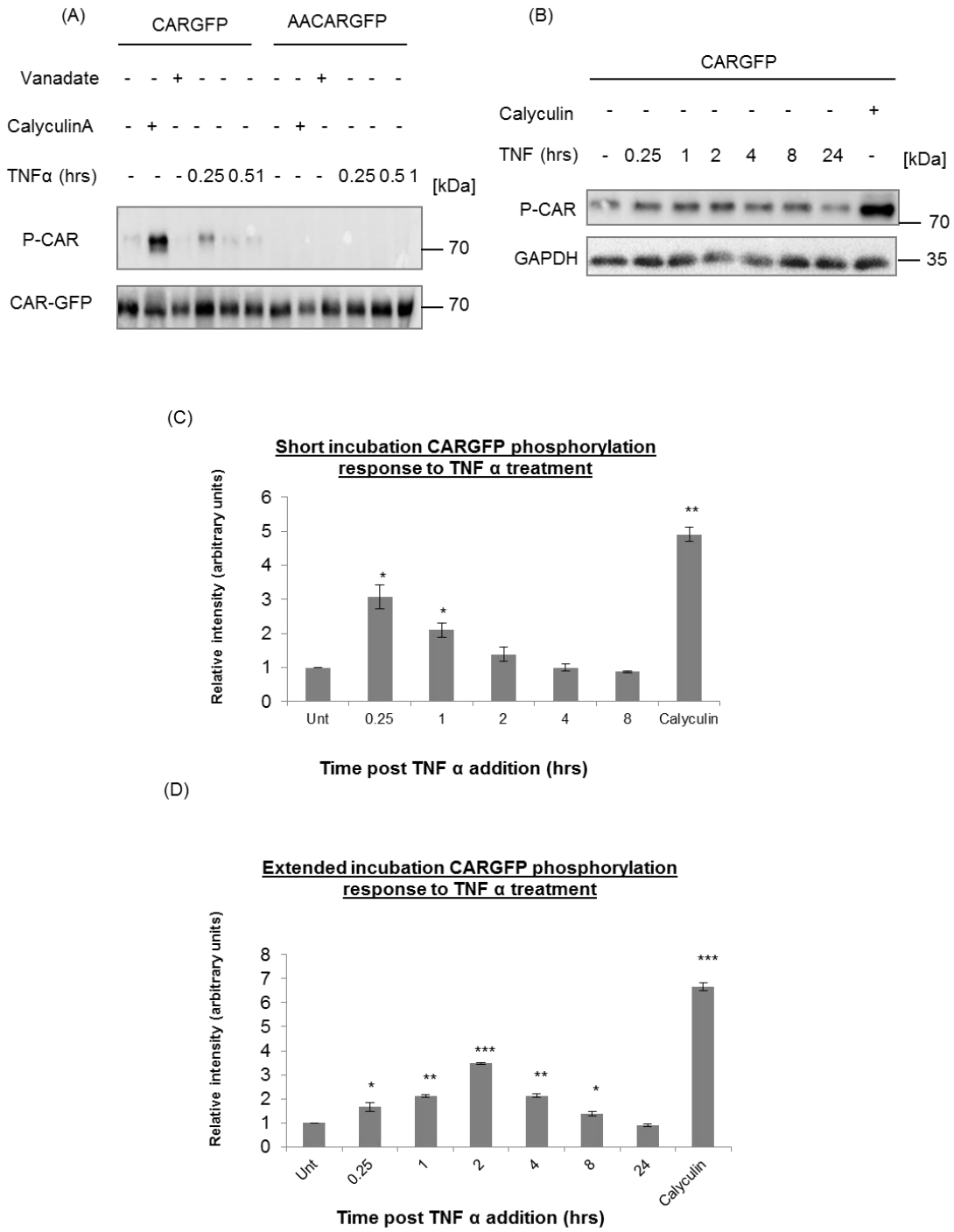


Figure 3-5 CAR phosphorylation following the addition of TNF α at the serine/threonine sites. A) Western of immunoprecipitated CAR-GFP from HBEC lysates treated as indicated with either 30 μM Pervanadate, 0.5 mM Calyculin A or 10ng/ml TNF α. Cells were grown for approximately 30 hours prior to treatment. Western blots were probed with the CAR specific serine/threonine phospho-specific antibody showing the strongest band at 15 minutes in response to TNF α. (N=3) B) Western blot of HBEC lysates treated as indicated with 10ng/ml TNF α. Cells grown for 72 hours before treatment with the strongest band at 2 hours in response to TNF α (N=3). C&D) Relative intensity of P-CAR bands from westerns with a short growth period of (30 hours-C) and a long growth period (72 hours -D) - Error bars are SEM. *=p<0.05, **=p<0.01 ***=p<0.005 compared to untreated sample. N=3.

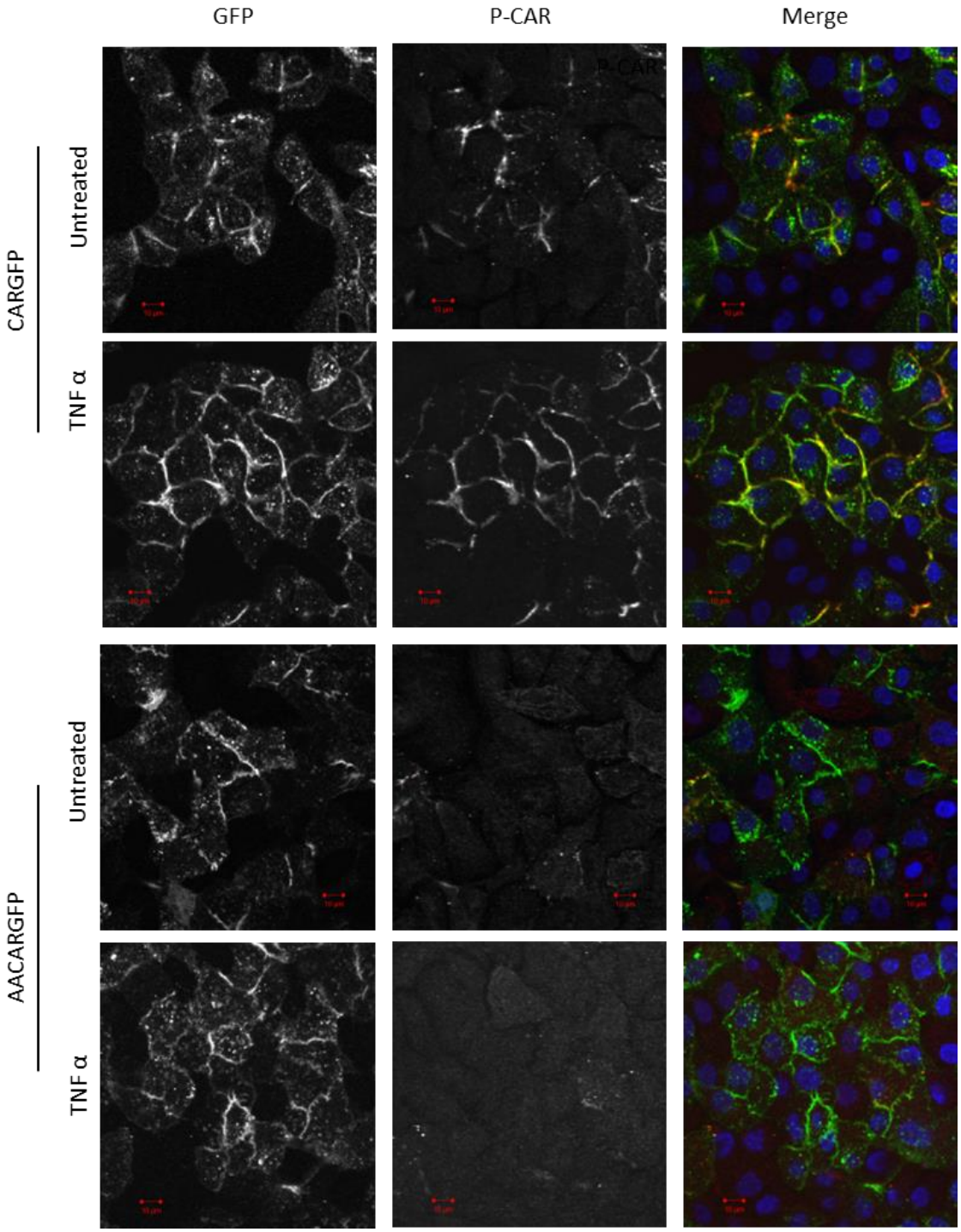


Figure 3-6: Imaging of CAR phosphorylation following the addition of TNF α at the serine/threonine sites. Confocal images of CAR-GFP and AACARGFP cells following treatment with 10ng/ml TNF α for 15 minutes using either GFP or the serine/threonine phospho-specific antibody (representation of 3 experiments) (Scale bar 10μm).

3.2.4 Development of tyrosine phosphorylation specific CAR antibodies and response to TNF α

An antibody (p-tyr269 CAR ab) was also raised against the tyrosine phosphorylation site at position 269 on the CAR cytoplasmic tail using the peptide sequence EEK(pY)EKEV-C. This was a rabbit derived polyclonal antibody developed in collaboration with Perbioscience (Thermofisher). This targeted the KKRREEK**Y**EK sequence identified on the cytoplasmic tail of CAR as a possible site for tyrosine phosphorylation (figure 1.7).

Sera taken from initial bleeds from the two inoculated rabbits showed no detection of a specific tyrosine phosphorylation band in response to the positive control vanadate (a tyrosine phosphatase inhibitor) (figure 3.7a). Western blotting of the same samples with a commercial pan-phosphotyrosine antibody showed robust tyrosine phosphorylation indicating the positive control had caused tyrosine phosphorylation. This poor response is consistent with the ELISA data supplied by Perbioscience (Thermofisher) which demonstrates that there was less of an antibody response in comparison to the p- thr290/ser293 CAR ab (figure 3.7b).

Given that the rabbits had failed to mount an adequate antibody response to the first inoculation rabbit PA5832 underwent a second prolonged stimulation with the peptide. To test the antibodies function CAR-GFP was immunoprecipitated from vanadate and TNF treated HBEC lysates and western blotted using the p-tyr CAR ab (figure 3.7c). Following the extended inoculation the resulting antibody did detect a band at the appropriate molecular weight for phosphorylated CAR in response to the positive control for tyrosine phosphorylation, vanadate. There was, however, no tyrosine response to TNF α stimulation (figure 3.7c) implying that its effects were specific to the serine/threonine site. The mutant tyrosine site

cell lines were also used in this experiment. The Y2ECAR-GFP cells were those that had the CAR cytoplasmic tail altered to ensure that they appeared permanently phosphorylated at the tyrosine site, whereas on the Y2FCAR-GFP cells it was altered to prevent phosphorylation of the tyrosine site. As outlined in the chapter introduction mutations of tyrosine phosphorylation sites do not necessarily produce the intended changes. With these cells the results suggest that this is the case as the phospho-mimic cells (Y2ECAR-GFP) in fact behave as if they are not able to be phosphorylated at the tyrosine site (figure 3.7c).

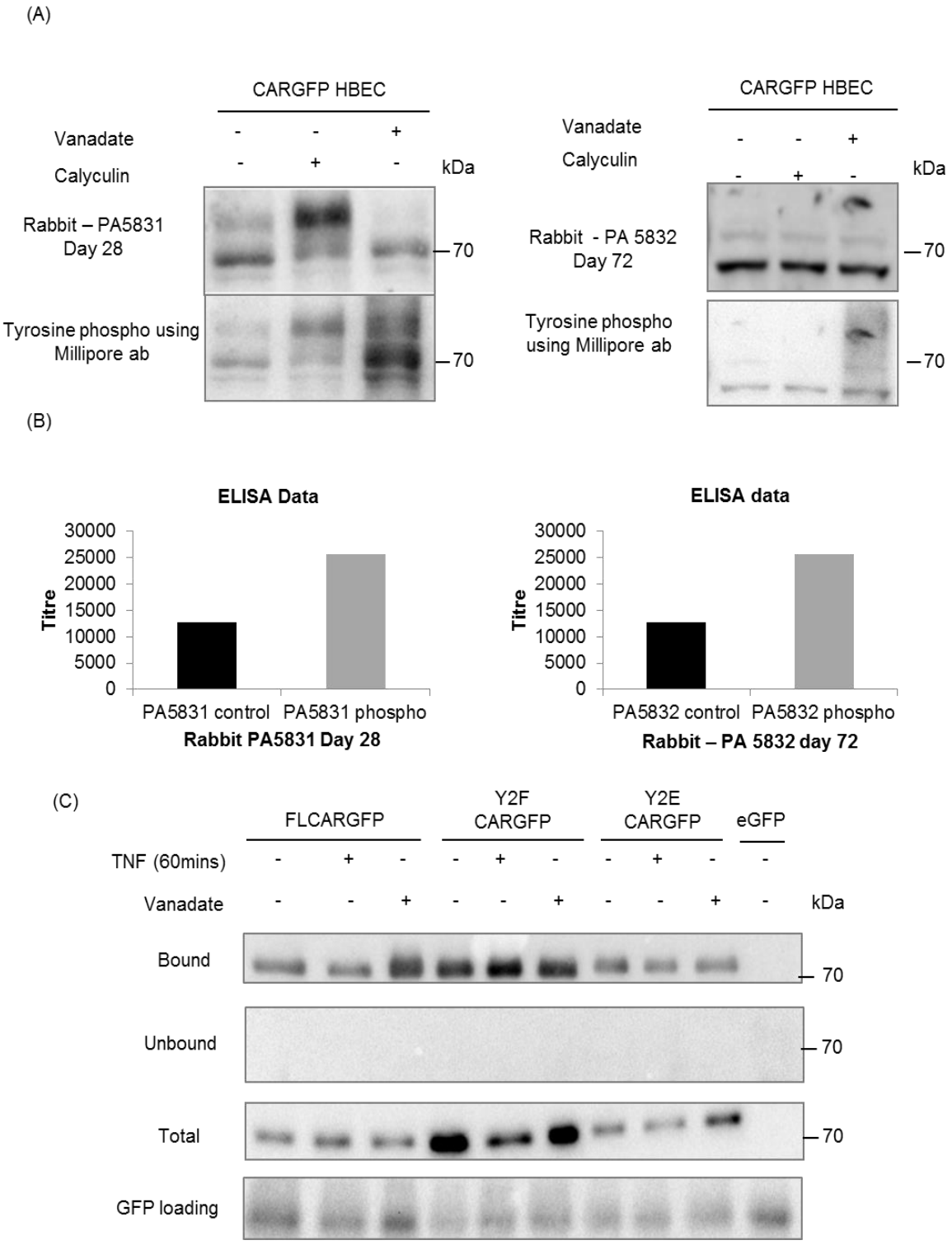


Figure 3-7 Phospho-tyrosine CAR antibody development and response to cytokine stimulation.

A) Initial western response using the bleed day with the strongest ELISA response (n=1). B) ELISA values from the two rabbits. C) Western blot of immunoprecipitated CAR-GFP from HBEC lysates treated as indicated with either 30 μM Pervanadate or 10ng/ml TNF α. Cells were grown for approximately 30 hours prior to treatment. Western blots were probed with the purified CAR specific phospho-tyrosine antibody from rabbit PA5832 following a prolonged inoculation period. A response is seen in the third bound lane in response to vanadate in the CAR-GFP cell line but not with TNF α (n=3).

3.2.5 CAR response to cytokines

TNF α has a broad inflammatory effect on the bronchial epithelium, which includes disruption of junctions and stimulation of alternative cytokine secretion (Hardyman *et al*., 2013, Mazzon and Cuzzocrea, 2007, Turner, 2009, Al-Sadi *et al*., 2009, Al-Sadi *et al*., 2008). However, the inflammatory process is not driven by TNF alone. Instead a multitude of complex interactions occur between a variety of cytokines and cells. As discussed in the chapter introduction multiple alternative cytokines play a role in tight junction protein function. Alternative cytokines were therefore trialed to determine whether TNF α was unique in causing CAR phosphorylation.

Treatment with IL-5 promoted phosphorylation of the serine/threonine site on CAR (figure 3.8 a & b). This was not as strong as that seen with TNF but was consistent and reproducible. IL-5, like TNF α, is a chemoattractant and stimulant for eosinophils in ulcerative colitis (Lampinen *et al*., 2001). Importantly IL-5 is also known to be upregulated in patients with asthma, in particular in those with more severe disease (Peters *et al*., 2014). These patients are also those known to have an altered epithelial-mesenchymal trophic unit. However, there have not been previous reports showing a direct effect on epithelial cells and therefore this observed effect on CAR is novel for both its phosphorylation of this protein and its impact on epithelial cells. PKC δ is known to phosphorylate CAR and interestingly we also observed phosphorylation of PKCδ downstream of IL-5 (fig3.8a).

Additionally the cytokines IL-13, IL-1β, IL-17 and IL-8 were tested to determine whether they also induced phosphorylation of CAR (figures 3.8 c, d, e and f respectively). These cytokines have all been shown drive an inflammatory response, with evidence of IL-1β, IL-13 and IL-17 in particular causing epithelial barrier dysfunction through tight junction impairment (Al-Sadi *et al*., 2008, Turner,

2009, Schulzke *et al*., 2009). However, none of these cytokines promoted the phosphorylation of the serine/threonine phospho-sites on the cytoplasmic tail of CAR (Figure 3.8c-f).

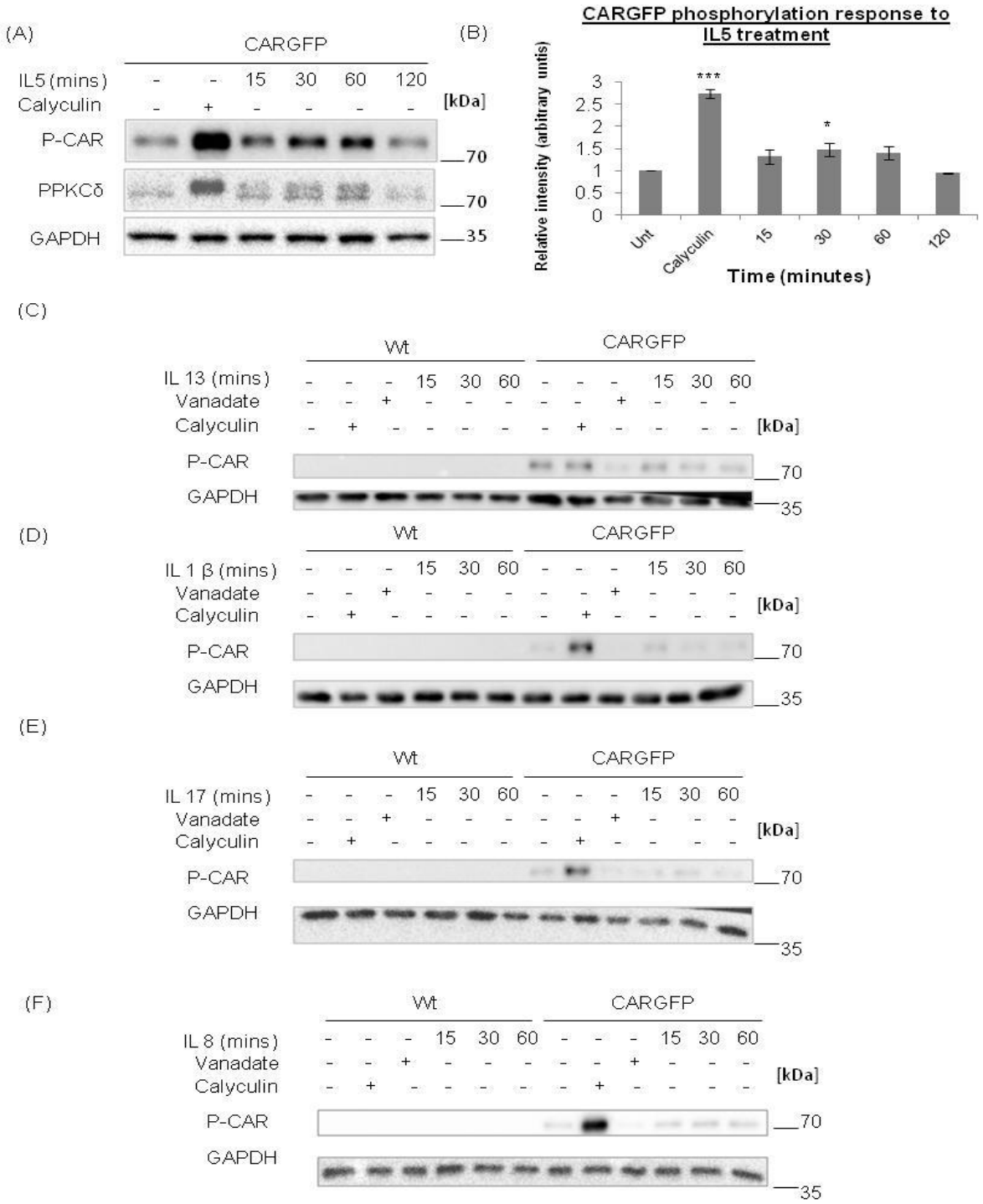


Figure 3-8 CAR response to alternative cytokines.
A) IL-5 stimulates ser/thr CAR phosphorylation in a similar time dependent and PKCδ fashion as TNF α. (n=3) B) The graph shows the relative signal intensity in response to IL-5 (25ng/ml) in 3 independent experiments. Error bars are SEM. *=p<0.05, **=p<0.01 ***=p<0.005 compared to untreated samples. C) No response seen to IL-13 (25ng/ml) presence (n=2). D) No response seen to IL1β (25ng/ml) presence (n=2). E) No response seen to IL-17 (25ng/ml) presence (n=2). E) No response seen to IL8 (5ng/ml) (n=2).

3.2.6 PKCδ is responsible for CAR phosphorylation in response to TNF α stimulation

The role of PKC δ in the phosphorylation of the serine/threonine site has already been established (Morton *et al*., 2013). This demonstrated that the absence of PKC δ resulted in loss of serine/threonine phosphorylation of the CAR cytoplasmic tail in response to Phorbol 12,13-dibutyrate (PDBu; an activator of cPKC's) or calyculin A . PKC δ is also activated in response to TNF activation of TNFR1 in neutrophils (Kilpatrick *et al*., 2006, Kilpatrick *et al*., 2002). Given the established role for PKC δ in CAR phosphorylation, the activation of PKC δ was studied in response to TNF α treatment (figure 3.9a). PKCδ phosphorylation showed a time dependent response to TNF α with kinetics that mirrored the phosphorylation of CAR. To determine whether PKCδ has a direct effect on CAR phosphorylation in response to TNF, siRNA to PKCδ was used to transiently reduce PKCδ expression in HBEC. PKCδ knockdown resulted in reduced CAR phosphorylation in response to TNF α stimulation compared with TNF α alone controls (Fig3.9B). This finding was confirmed using confocal microscopy, as a p-ser/thr CAR response at cell junctions to TNF α treatment was seen at sixty minutes, and this was lost when PKCδ was depleted (figure 3.10c). Taken together, these data support a role for PKCδ in controlling phosphorylation of the CAR cytoplasmic tail in response to TNF α.

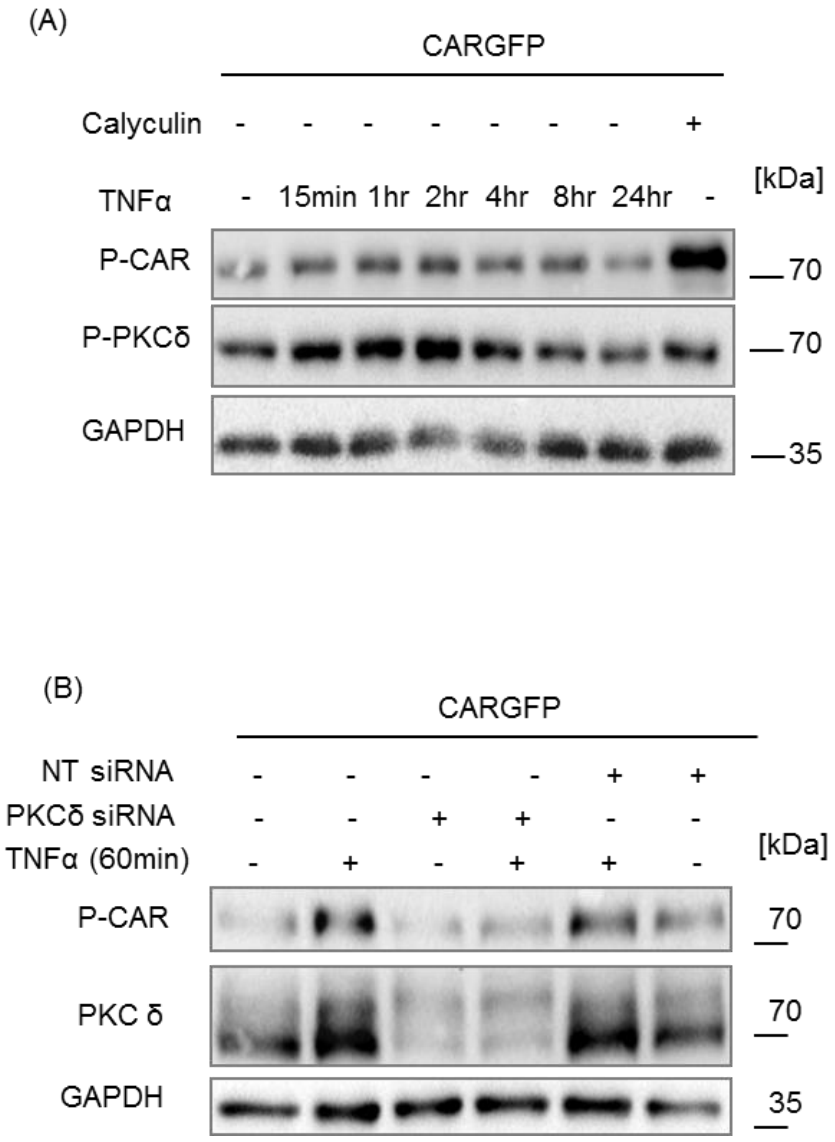


Figure 3-9 PKC δ phosphorylates CAR downstream of TNF α.
A) Western blot analysis of phospho-CAR and phospho-PKCδ after treatment with 10ng/ml TNF α for the indicated times. Western blots were also probed for GAPDH as a loading control. (N=3) B) Western blot analysis of phospho-CAR in CAR-GFP HBEC expressing PKCδ siRNA or a non-targeted control (NT). Cells were treated with 10ng/ml TNF α for 60min where indicated. Western blots were probed for phospho-CAR, PKCδ and GAPDH as a loading control. (N=3)

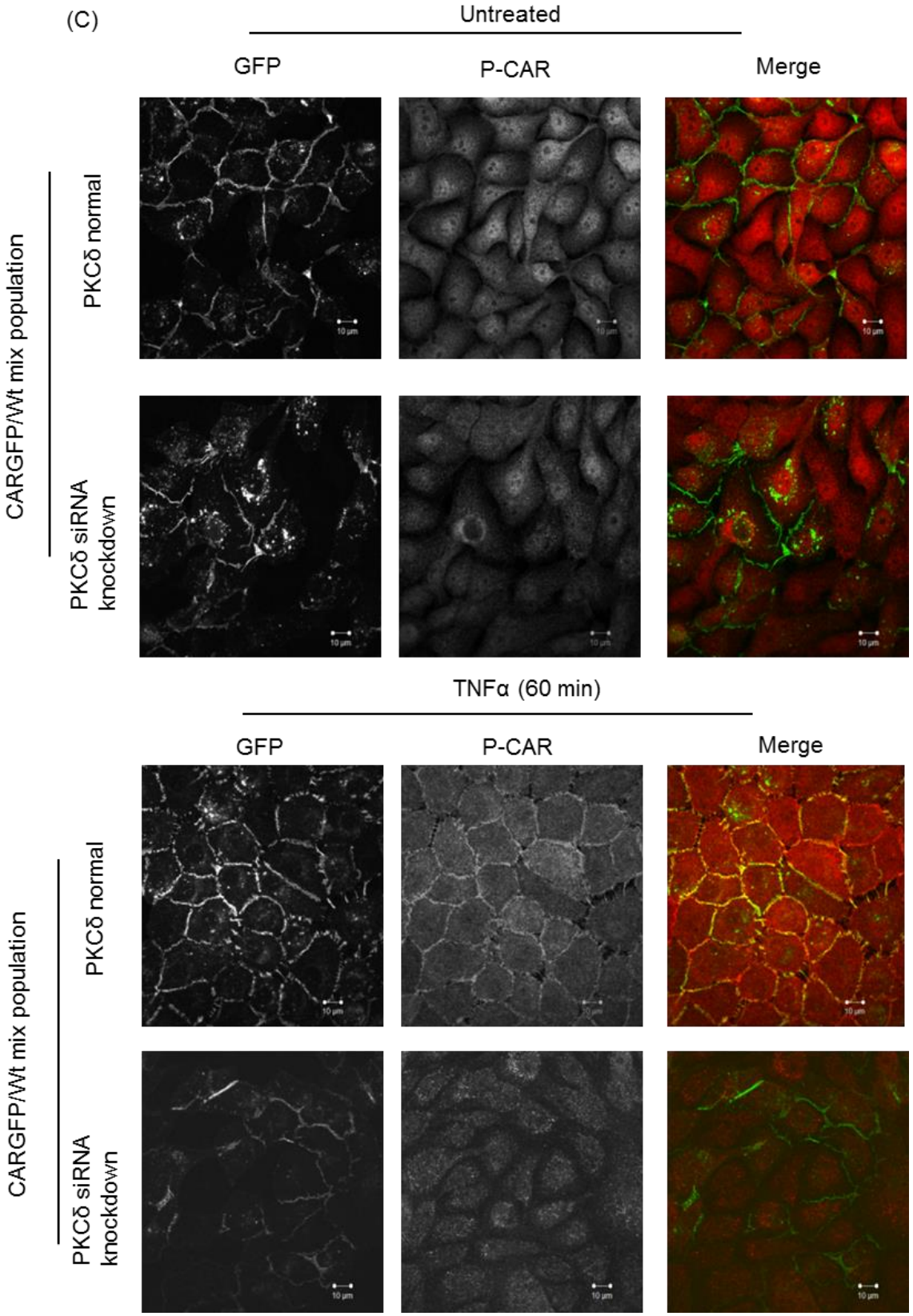


Figure 3-10 PKC δ phosphorylates CAR downstream of TNF α (imaging). Representative confocal imaging of 3 separate experiments with CAR-GFP (green) and PKCδ (red) expressing PKCδ siRNA either untreated or treated with TNF α in comparison to CAR-GFP (scale bar 10μm).

3.2.7 PI3K inhibition leads to loss of PKCδ phosphorylation and therefore CAR phosphorylation in response to TNF α

PI-3 kinase has previously been shown to be required for TNF α mediated PKCδ activity in neutrophils (Kilpatrick *et al*., 2002). The activation of PI3K by alternative cytokines has also been shown to affect tight junction function in epithelial cells, in particular INF γ (Boivin *et al*., 2009, Al-Sadi *et al*., 2009). We therefore hypothesised that PI3K may be required for PKCδ mediated CAR phosphorylation downstream of TNF α.

To test this hypothesis, HBEC cells were treated with the PI3K inhibitor LY294002 (figure 3.11a). This experiment was performed over a time course of TNF treatments with the cells incubated for an extended period and therefore expected to have a prolonged stimulation period before causing phosphorylation. Following the addition of LY294002 there was a loss of both CAR and PKC δ phosphorylation in response to TNF α (figure 3.11). The relative effect of this reduction in three separate experiments is most pronounced between two to four hours reflecting the longer time course for activation seen in HBEC cells grown for an extended period (figure 3.11b). These data indicate that PI3K supports the phosphorylation of the serine/threonine site on the cytoplasmic tail of CAR and also that this effect occurs upstream of PKCδ as its inhibition also leads to reduction of PKCδ activation.

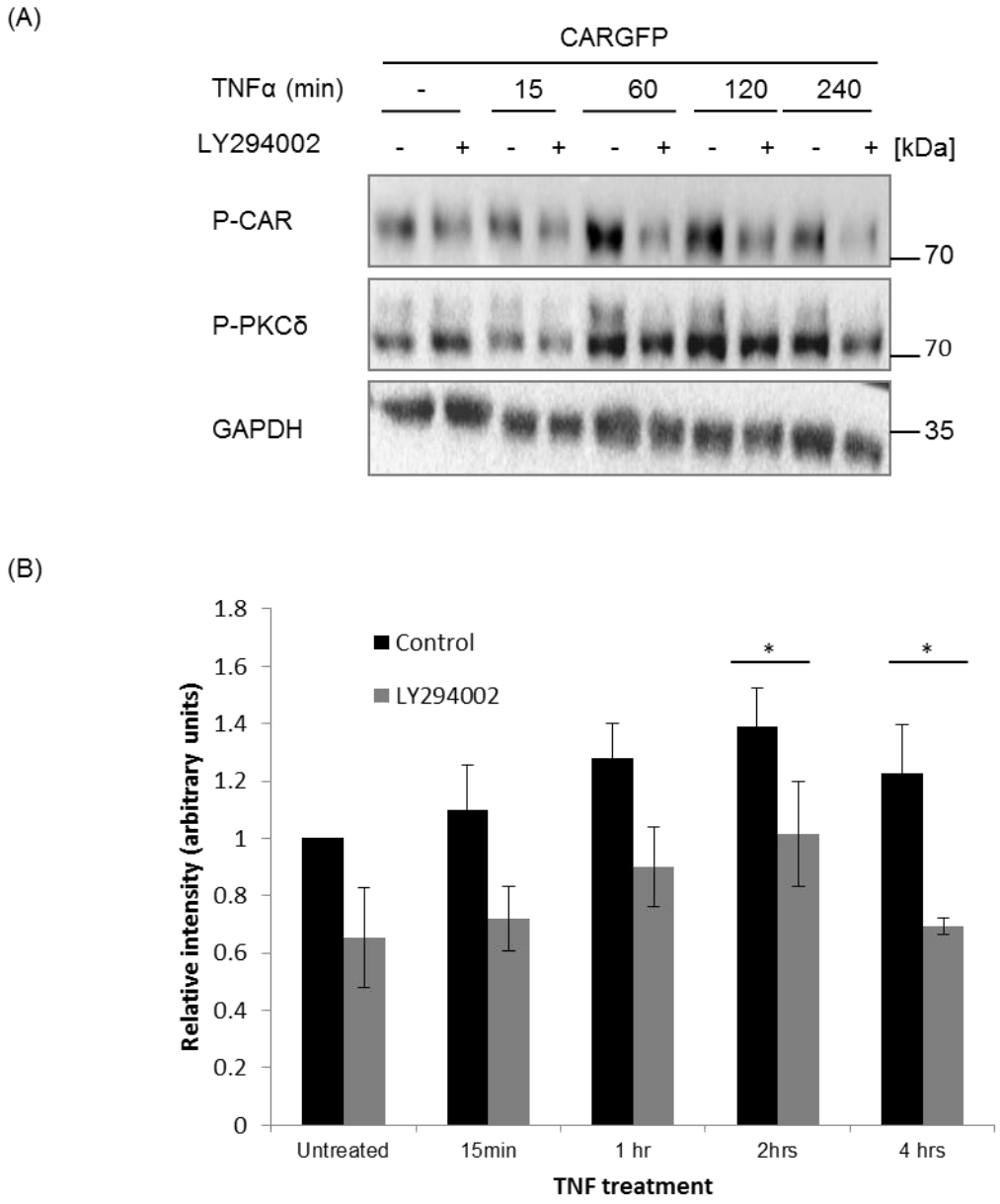


Figure 3-11 PI3K inhibition causes loss of both PKCδ phosphorylation and CAR phosphorylation in response to TNF α.

A) Western blot analysis of phospho-CAR and phospho-PKCδ after pre-treatment with 10μM/L LY294002 for 2 hours where indicated and further treatment with 10ng/ml TNF α for the times shown. Western blots were also probed for GAPDH as a loading control. B) Relative intensity of P-CAR bands from westerns - Error bars are SEM. *=$p<0.05$. N=3.

3.2.8 NFκB activation downstream of TNF is unaffected by CAR

Alternative cell signaling pathways known to be stimulated by TNF α were also investigated. Binding of TNF α to its cell receptor TNFR1 on epithelial cells results in the recruitment of a complex of proteins to the cell membrane to drive intracellular signaling, including TRADD and RIP1, which in turn cause signal transduction via NFκB (Kilpatrick *et al*., 2006, Micheau and Tschopp, 2003). NFκB is rapidly transported from the cytoplasm to the nucleus to trigger gene transduction. Therefore to determine whether this process was altered by the presence of phosphorylated CAR, CAR-GFP HBEC and WT HBEC were treated with TNF α and fixed at two different time points. These cells were then fixed and stained for NFκB to identify its sub-cellular location. In both cell types, NFκB moved to the nucleus by thirty minutes with no alteration seen regardless of whether CAR was overexpressed in the cells (figure 3.12), indicating that activation of this pathway is unaffected by the presence of overexpressed CAR.

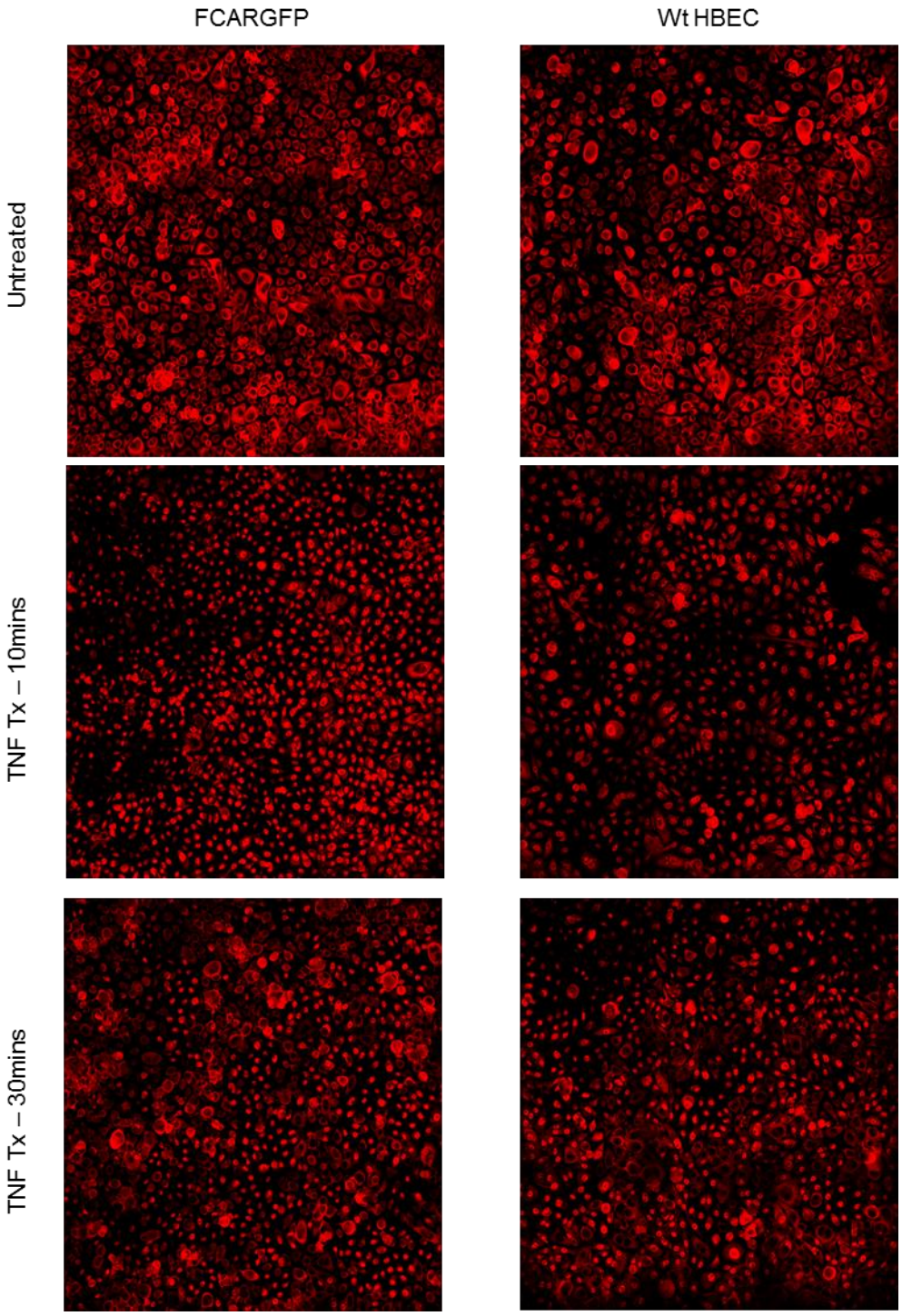


Figure 3-12 NFκB signaling is unaffected in the presence of CAR. Confocal imaging of NFκB (Alexa 568 secondary antibody; red) in Wt HBEC and CARGFP HBEC treated with 10ng/ml TNF for the times indicated before fixation. NFκB shows nuclear staining when activated in response to TNF α (Representative imaging of 3 separate experiments).

3.2.9 MAPK signalling in response to TNF is unaffected by CAR overexpression

TNF α is also known to activate the MAP kinases following recruitment of the TRADD based protein complex to the cell membrane. Multiple MAPK pathways exist following TNF treatment including p38 MAPK and Extracellular Signal-Related Kinases (ERK) and their responses are seen to be cell specific (Kant *et al*., 2011).

To assess responses in this pathway, western blotting analysis of both ERK and p38 phosphorylation in response to TNF α was performed in both CAR-GFP HBEC and WT HBEC (figures 3.13 a, b). No stimulation of ERK by TNF α was detected in these cells and this was unchanged by overexpression of CAR (figure 3.13a). The timing points for ERK phosphorylation were used to correspond to the equivalent CAR phosphorylation events. The ERK response itself can be varied by cell type and this may have underestimated the ERK response as this can occur very rapidly. It has been shown to have a more gradual response in mammalian cells (Aoki et al., 2011) and the aim of the experiment was to ensure that the additional presence of phosphorylated CAR was not altering this signalling pathway. The presence of TNF α, however, did lead to p38 phosphorylation, but this was not significantly different in HBEC overexpressing CAR (figure 3.13 b). The response by these MAP kinases to TNF α is not a novel finding but reinforces the NFκB results and further supports that CAR overexpression does not cause general alterations in multiple signalling pathways.

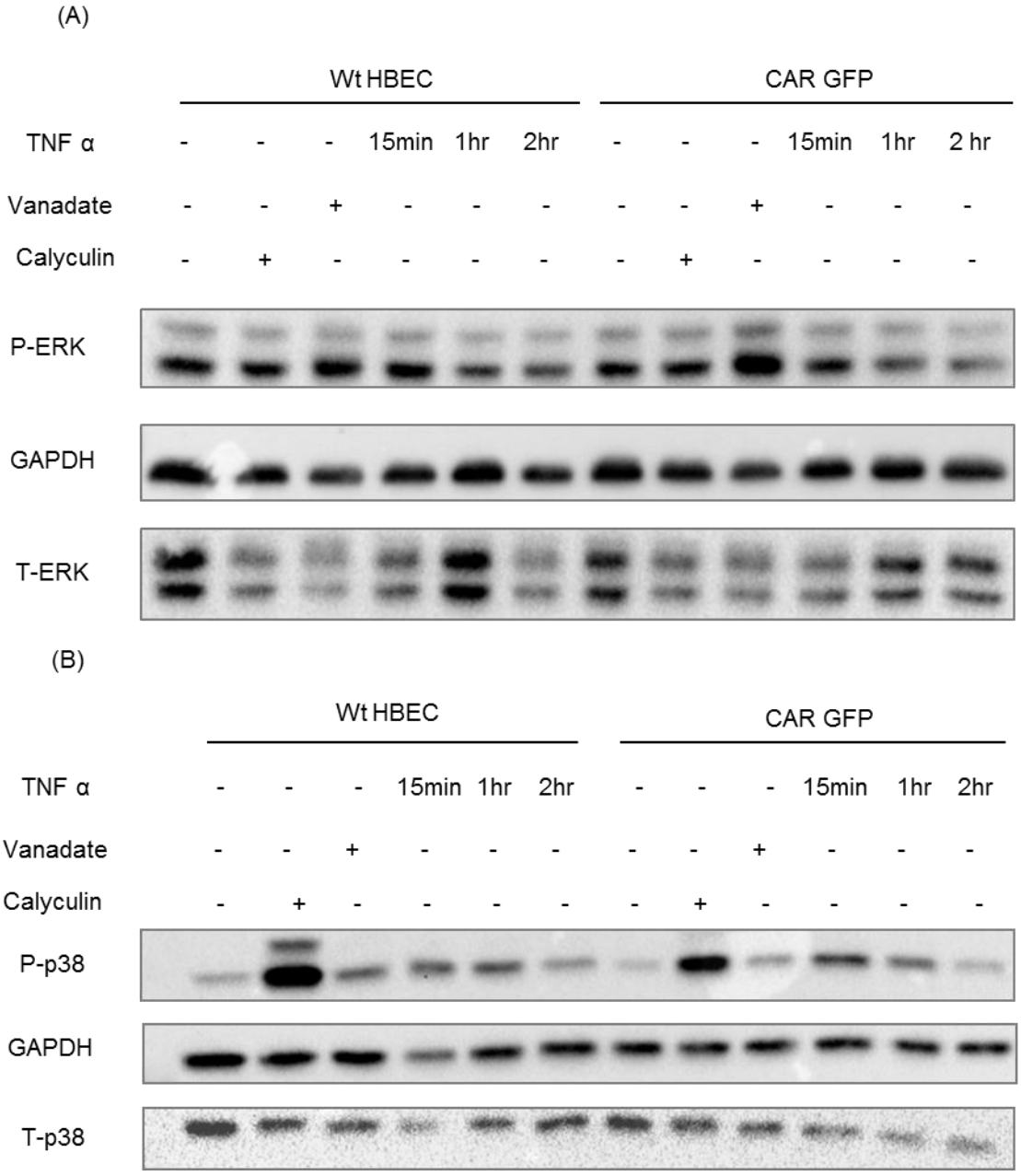


Figure 3-13: MAPK signaling unaffected in the presence of phosphorylated CAR.
A) P-ERK activation in CAR-GFP HBEC and Wt HBEC in the presence of 10ng/ml TNF α and phosphatase inhibitors calyculin A and vanadate. Two bands seen at 42 and 44 kDa. (N=5) B) P-p38 activation in CAR-GFP HBEC and Wt HBEC in the presence of 10ng/ml TNF α and phosphatase inhibitors. (N=5)

3.2.10 The disruption of CAR with adenovirus 5 fibre knob leads to the loss of CAR phosphorylation in response to TNF α

The initial work shown indicates that CAR can be phosphorylated at the serine/threonine sites on its cytoplasmic tail. However, whether this occurs at cell junctions or within intracellular vesicles was not determined. In stable cell monolayers, CAR forms homodimers with other CAR molecules from adjacent cells in tight junctions. The adenovirus 5 fibre knob (Ad5FK) binds competitively to the extracellular domain of CAR (Santis *et al*., 1999, Kirby *et al*., 2000). This binding has a significantly greater affinity than the CAR extracellular domain has with itself and thus the presence of Ad5FK causes disruption of CAR homodimerisation at cell junctions.

Western blot analysis was performed on lysates of stable CAR-GFP HBEC monolayers treated with Ad5FK prior to the addition of TNF α. The addition of Ad5FK with the resulting loss of CAR homodimerisation at cell-cell tight junctions was associated with the inability of TNF α to cause p-ser290/thr293 of the cytoplasmic tail of CAR (figure 3.14). This implies that CAR homodimerised across adjacent cell-cell contacts in order for it to be phosphorylated.

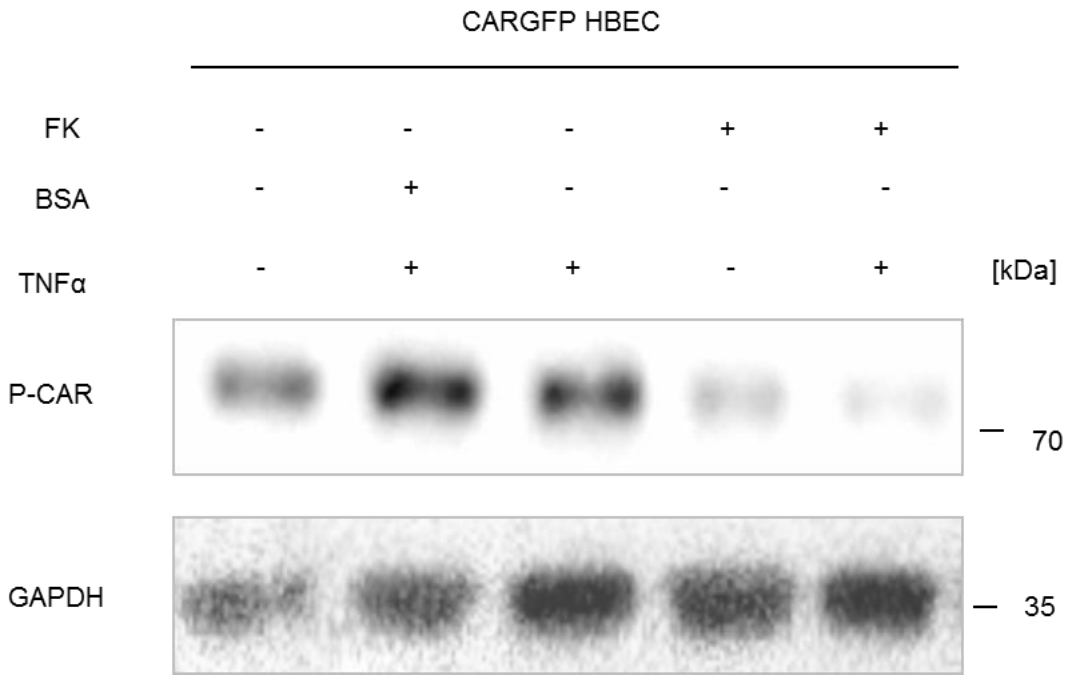


Figure 3-14: Fibre knob associated disruption of CAR homerdimerisation leads to loss of TNF α driven CAR phosphorylation. Western blot analysis of CARGFP HBEC treated with 10ng/ml TNF α and 100µg/ml Ad5FK or BSA 100µg/ml control as indicated, probed for phospho-CAR and HSC-70 as a loading control.

3.3 Discussion

This chapter set out to establish whether the presence of cytokines associated with tight junction disruption and respiratory inflammation are involved in the phosphorylation of CAR.

3.3.1 CAR phosphorylation in response to cytokines

CAR has been shown to be involved in the immune response by acting as a binding partner for proteins on neutrophils and other immune cells during their passage through epithelial layers (Verdino *et al*., 2010, Witherden *et al*., 2010). The findings presented here, that TNF stimulates CAR phosphorylation, provides a novel mechanistic insight into control of CAR in the inflammatory cascade. TNF α has been shown to interact with alternative tight junction proteins resulting in their disruption as well as activating protein kinases that are responsible for phosphorylation events during the immune response, therefore suggesting that CAR phosphorylation has a physiologically relevant function (Al-Sadi *et al*., 2009, Schmitz *et al*., 1999, Coyne *et al*., 2002). This is supported by the finding that the p-ser290/thr293 phosphorylation is a rapid time dependent response that was lost within a few minutes to hours, depending on the density of the cells being treated. This rapid phosphorylation response suggests that CAR plays a more active role in leucocyte transmigration than previously thought as it happens in a similar timeframe as leukocyte movement occurs in response to an inflammatory stimuli (Sarris *et al*., 2012).

A further tyrosine at the 269 position was studied to determine whether this may be an additional phosphorylation site. Interestingly the data showed that this site was not phosphorylated in the presence of TNF α. This is a noteworthy finding given the previous study by Angelini *et al (2006)* in which pulmonary vascular

endothelial cells were shown to exhibit high active Src family kinases following the addition of TNF α. This in turn caused phosphorylation of alternative junctional proteins including vascular endothelial cadherin and P120 catenin (Angelini *et al*., 2006). This difference in the site of phosphorylation stimulated by TNF α may be a reflection of the difference in cell type studied, namely epithelial as opposed to endothelial cells or may reflect a different role for CAR in cell junctions.

IL-5 has not previously been indicated in epithelial cell signalling. Classically IL-5 is seen as a TH2 cytokine that acts as an eosinophil chemo-attractant and is therefore associated with diseases such as asthma (Peters *et al*., 2014). Intriguingly it has, though, been shown to activate PKCδ (Bankers-Fulbright *et al*., 2001). In CAR-GFP HBEC, the pathway for both TNF α and IL-5 driven phosphorylation of CAR was also via activation of PKCδ. This may therefore provide a link between these two cytokines given that IL-5 has not previously shown to be active in cells that are not part of the immune system. It was also established that PI-3K was required as an intermediate step in the activation. As described, both of these kinases are known to be activated in response to TNF α and IL-5. Separately it was also shown that CAR was phosphorylated via PKCδ but this work is the first to show that the two processes are connected and that they occur in epithelial cells.

Alternative cytokines were tested to determine if they would also induce a similar response in CAR. As outlined in the introduction multiple cytokines have been identified as triggering disruption of tight junction proteins including IFN γ, IL-1β, IL-13 and IL-17 but from this data do not cause phosphorylation of the serine/threonine site on the cytoplasmic tail of CAR. This selectivity is interesting given our current knowledge of the effect of these alternative cytokines. IFN γ did

not affect CAR in this cell model but is known to activate the PI3K pathway which our results show activates CAR phosphorylation (McKay *et al*., 2007, Boivin *et al*., 2009). This difference could reflect the difference in cell types used in these studies; gastrointestinal epithelial cells versus the respiratory epithelium. Further work with gastrointestinal epithelial cells shows that in the presence of IFN γ there is an increased turnover of tight junction proteins including JAMA (Bruewer *et al*., 2005) which may suggest that INF γ is having longer-term effects on protein localisation rather than triggering short term changes to tight junction proteins through phosphorylation. Therefore given that these cytokines are not driving CAR phosphorylation at the cytoplasmic tail the response to TNF α and IL-5 is not a simple reaction to tight junction impairment or alteration alone but is instead directly related to their presence.

3.3.2 CAR at the cell membrane

The second key aspect of these findings is that this phosphorylation effect occurs when CAR is at the cell membrane. The basis for investigating this phosphorylation effect was our recent work (Morton *et al* 2013) which showed that this site on the cytoplasmic tail of CAR can be phosphorylated leading to alterations in cell-cell junction protein localisation. These adhesion proteins, in particular E-cadherin, have also been found to be altered following cytokine stimulation as part of the immune response (Al-Sadi *et al*., 2009). The previously identified CAR phosphorylation events were found to occur at both junctions and recycling vesicles in association with disruption to the junctions (Morton *et al*., 2013). This process of CAR phosphorylation was related to the localisation of junction proteins, as E-cadherin was recycled from the cell membrane when CAR was not phosphorylated. Confocal images presented in this chapter indicate that CAR phosphorylation is specifically occurring at the membrane in response to

TNF α. This is significant, as CAR has long been established as playing a role in the formation and stability of tight junctions via homerdimerisation of its extracellular domain (Cohen *et al*., 2001). As previously described this position at the cell membrane also places CAR in the position where it can bind with leucocytes implicating it in the immune response. Therefore the fact the phosphorylation response to TNF α occurs only at the membrane implies that it is of physiological relevance, possibly by ensuring that CAR is still in a position to act on the transmigrating leucocytes. Its physiological relevance was reinforced by that fact that although these effects were demonstrated in an immortalised cell line overexpressing CAR, the downstream pathways for TNF α signalling were not being altered by the presence of phosphorylated CAR and thereby implying that CAR overexpression is not altering the overall mechanics of the cell.

4 Inflammation leads to CAR phosphorylation and immune cell migration *in vitro*

4.1 Introduction

The previous chapter showed that the serine/threonine site on the cytoplasmic tail of CAR, when CAR is homerdimerised at cell junctions, is phosphorylated in response to TNF α. This phosphorylation is driven by PI-3K and PKCδ but does not result in an alteration in the expected intracellular signalling pathways associated with TNF α; NFκB, p38 and ERK. This chapter explores the physiological role the phosphorylation of CAR may have in inflammatory conditions.

4.1.1 CAR and the immune response

There is a growing body of evidence that the epithelial barrier in the lungs of patients with asthma and cystic fibrosis is disrupted through effects on both tight junctions (Xiao *et al*., 2011) and adherens junctions (Lambrecht and Hammad, 2012) (Coyne *et al*., 2002). As described previously CAR is a member of the epithelial junction complex and its expression is upregulated in chronic autoimmune inflammatory conditions in mice suggesting the role of CAR in cells is altered in response to inflammation (Ito *et al*., 2000). A mechanism for the role of CAR in this immune reaction has been suggested through more recent work that addressed CAR's interaction with other members of the JAM family (Verdino and Wilson, 2011, Witherden *et al*., 2010). As described previously CAR has a similar structure to other proteins in the JAM family and their extracellular components can hetero-dimerise. Some members of the family including JAM-A are also found on leucocytes as well as endothelial and epithelial cells. JAM-A disruption in leucocytes has long been known to lead to a reduction in leucocyte

trans-endothelial migration due to loss of homerdimerisation with JAM-A found on epithelial cells (Martin-Padura *et al*., 1998). The more recent work by Verdino *et. al*. and Witherden *et. al*. showed that CAR on the epithelial cell membrane binds to JAM-L on γδT cells and neutrophils (Witherden *et al*., 2010, Verdino *et al*., 2010, Zen *et al*., 2005). The γδT cells play a key role in host immunity and are resident in the epidermis (Jameson *et al*., 2002, Girardi *et al*., 2001, Sharp *et al*., 2005). These cells are the primary responders to epidermal insult thereby protecting against environmental insults such as infection, trauma and malignancy. Importantly where this interaction between CAR and JAM-L on epidermal and T cells respectively is disrupted skin healing is slowed (Witherden *et al*., 2010). Therefore JAM-L with its ligand CAR can been seen as a co-stimulatory receptor for γδT cells (Verdino and Wilson, 2011) which places CAR in the position to modulate the immune response. The nature of CAR's role in this process in particular needs to be established, as it's binding to JAM-L on immune cells may be a passive function but also have an active role through control of junction stability or through intracellular signalling. Therefore this complex interaction may be responsible for disease activity and also open the possibility for therapeutic modification.

4.1.2 Leucocyte Transepithelial Migration (TEpM) in response to cytokine stimuli

Leucocyte TEpM into luminal sites such as respiratory airways in response to inflammatory stimuli is a complex process that requires multiple molecular and cellular responses to occur in sequence, as outlined in the introductory chapter. It occurs in three steps: adhesion, migration and post migration (Zen and Parkos, 2003). CAR in its role as a ligand to JAM-L on immune cells has therefore been

implicated in the adhesion step and may also be necessary for successful migration.

This process requires a trigger, which is often provided by a cytokine. IL-1β, IL-8 and TNF α act as neutrophil chemo-attractants (Strieter et al., 1993, Strieter et al., 1992, Salva et al., 1996) and hence were tested as possible triggers for CAR phosphorylation in the previous chapter. These cytokines have also been implicated in the regulation of junctional proteins through modification of their expression and localisation to the cell membrane. This was shown for JAM, ZO-1 and ICAM-1 in response to TNF α and INF γ. (Coyne *et al*., 2002). This suggests a mechanism for feedback control of leucocyte movement.

Following the findings that CAR was phosphorylated at serine290 and threonine293 sites in response to TNF α we next sought to determine whether this process was integral to the previously established role for CAR in leucocyte migration.

4.2 Results

4.2.1 Localisation of TNF receptor and CAR in HBEC

In the previous chapter, it was established that CAR is phosphorylated at the serine290/threonine293 sites in response to TNF α stimulation in a time dependent fashion. This phosphorylation effect was mediated by PI-3K and PKCδ, however these results do not determine whether this process occurs with CAR in complex with the TNFR1 receptor or as part of a downstream signalling cascade (figure 4.1). TNF α signals predominantly through TNFR1 in epithelial cells as the alternative receptor TNFR2 is not known to be found in the epithelium (Speeckaert *et al*., 2012). Both CAR and TNFR1 are localised to the cell membrane and interact with other proteins at this site (Coyne and Bergelson, 2005, Park *et al*., 2014).

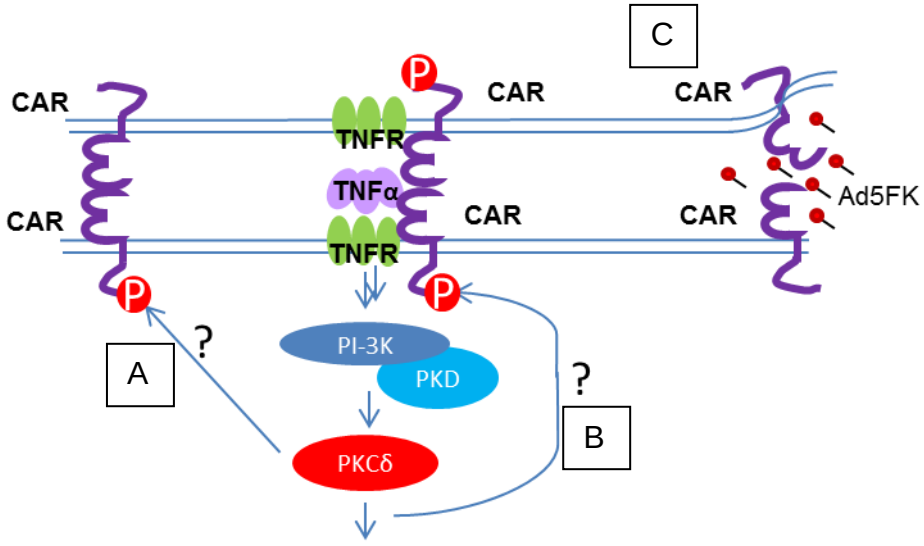


Figure 4-1: CAR phosphorylation at cell junctions: TNF signals through PI-3K and PKC which could act at a separate site at the cell membrane on CAR (A) or occur in complex (B). This phosphorylation effect though is lost if CAR is no longer homerdimerised at cell junctions (C).

To determine whether CAR and TNFR1 were in complex at junctions, confocal microscopy was performed to visualise their respective positions at the cell membrane in both resting conditions and in response to TNF α (figure 4.2b&c). The maximum intensity confocal microscopy projection images provided strong evidence that when at the membrane, the two receptors are not co-localised. This is not due to the over expression of TNFR1 in CAR-GFP cells as there are equal amounts of TNFR1 in the CAR-GFP cells as the WT HBEC (figure 4.2a).

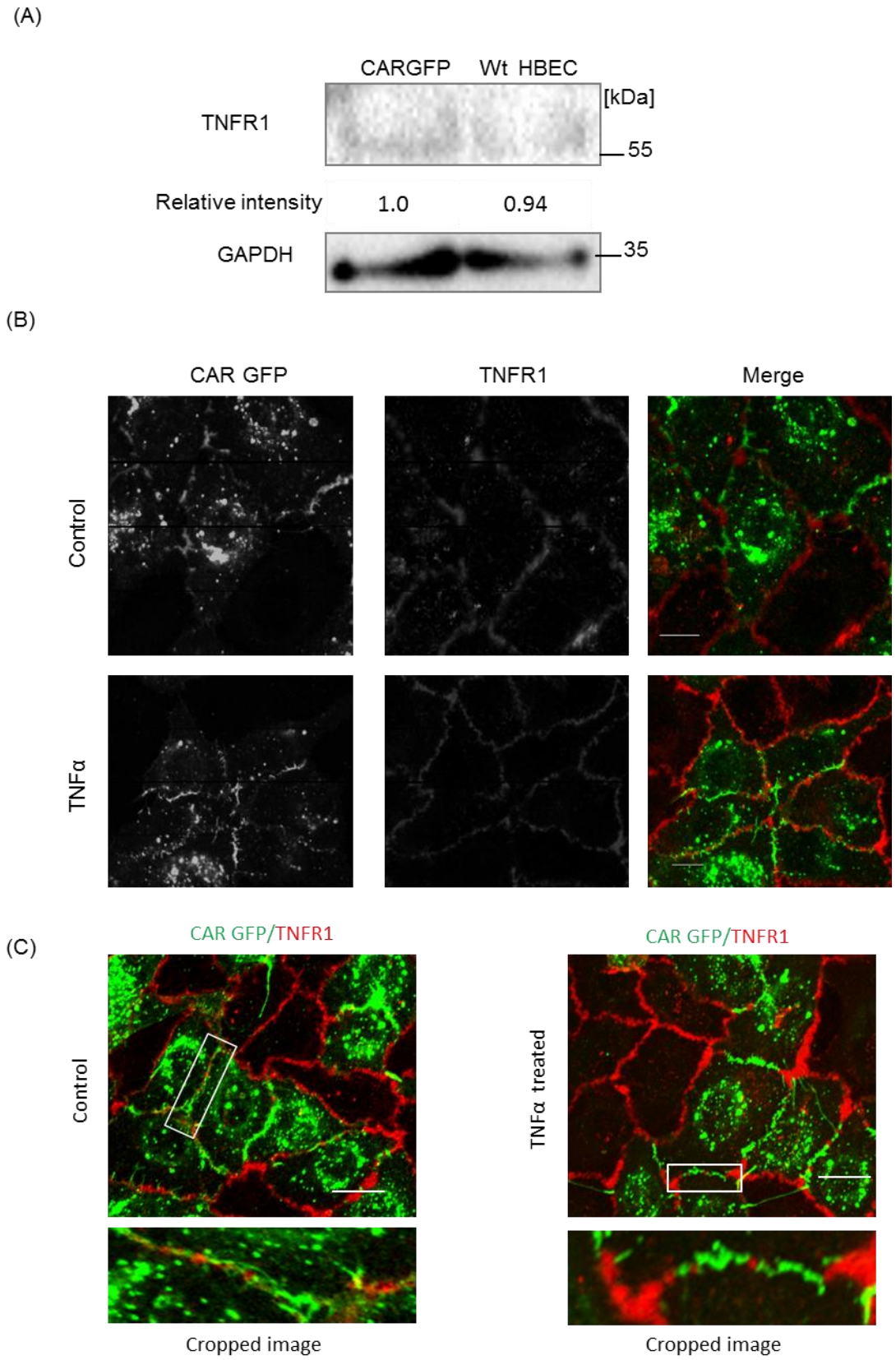


Figure 4-2: Localisation of TNF receptor and CAR in HBEC. A) TNFR1 levels in Wt HBEC and CARGFP HBEC. B) Confocal images of mixed populations of Wt HBEC and CARGFP HBEC with TNFR1 (red) and CAR (green) at cell junctions. C) Maximum intensity projection of the confocal images (TNFR1 – red and CAR – green). The white boxed areas are cropped and shown in the images below. Scale bars are 10um.

4.2.2 Functional effect of TNF α driven p-ser290/thr293 CAR phosphorylation

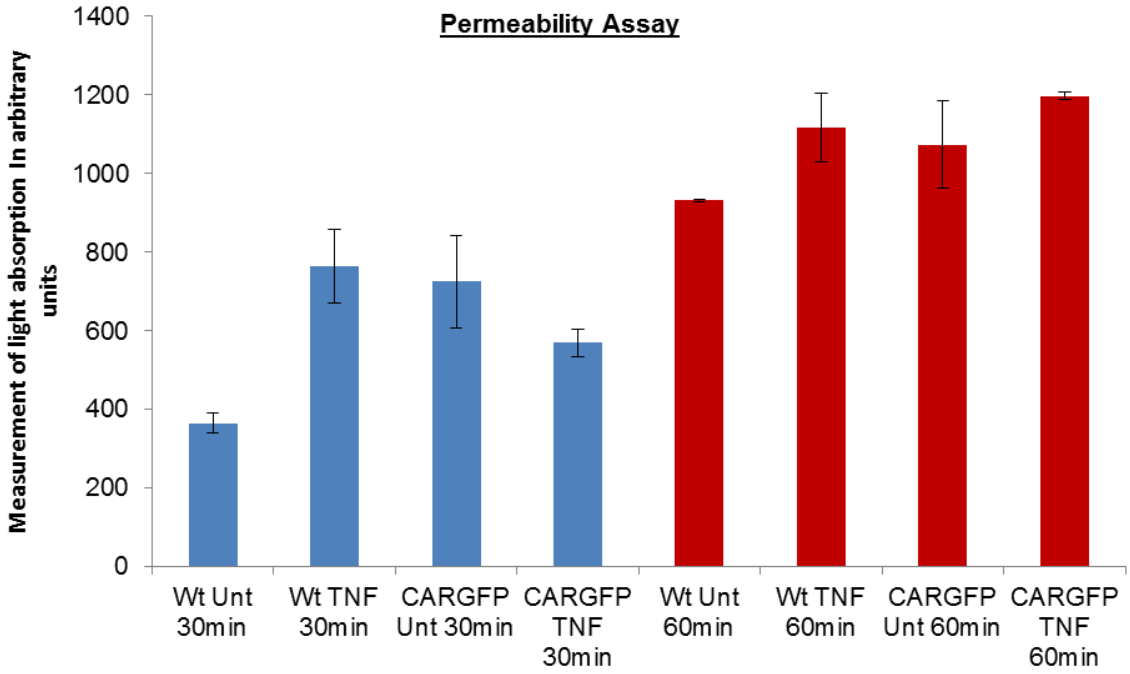
Increased epithelial permeability as a result of the presence of TNF α is a well-established phenomenon (Al-Sadi *et al*., 2009). The reasons for this response have been studied previously and multiple factors have been implicated, including loss of tight junction proteins (Al-Sadi *et al*., 2009, Ivanov *et al*., 2005) and increased cell death (Gitter *et al*., 2000a, Gitter *et al*., 2000b).

CAR has also been shown to influence permeability at tight junctions; CAR homodimerisation has been shown to be disrupted at tight junctions in T84 metastatic lung carcinoma cells by the addition of its soluble extracellular domain (CAR-ECD) to cell media (Cohen *et al*., 2001). These effects are organ specific though with loss of CAR leading to increased permeability in the heart myocytes but not in gut epithelial cells in knockout mice. (Pazirandeh *et al*., 2011)

Given the role both CAR and TNF α have been shown to play in the stability of the epithelial barrier it was therefore important to determine whether CAR was influencing the effect of TNF α on cell junction stability and permeability given the fact that CAR is being phosphorylated in its presence. Therefore permeability assays were undertaken as described in section 2.11. These assays were performed on stable confluent monolayers of either WT HBEC or CAR-GFP HBEC grown on transwells and either untreated or treated with TNF α. The amount of FITC-dextran passing through the monolayer is indicative of permeability between cells (figure 4.3 a,b). The reproducibility of the results in this model was highly sensitive to the formation of complete cell monolayers, which necessitated many repeats. The data is shown in two formats to aid interpretation. Firstly the average absolute values obtained from all of the

experiments are shown (figure 4.3a). The second figure shows the percentage change at each time point seen in those experiments where a realistic response to TNF α was seen. This removed two of the experimental repeats as there was no corresponding change in permeability with TNF which given the cytokines physiological effect likely reflects experimental error. Interestingly, the data shows that cells with high levels of CAR were less permeable in the presence of TNF α than those without, particularly at 30 minutes post TNF addition. This suggests that CAR plays a functional role in the response to TNF α by maintaining cell to cell contact. Given that the timing of this response coincides with phosphorylation effect seen in CAR in response to TNF α it may be that the two events are associated.

(A)



(B)

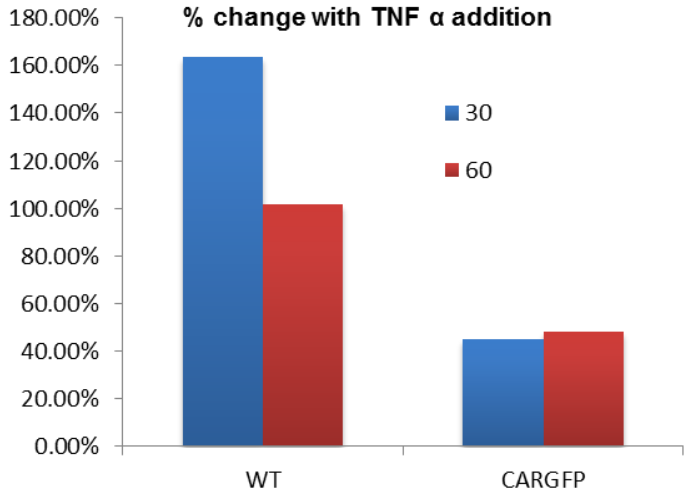


Figure 4-3: CAR plays a role in control of TNF-induced epithelial cell monolayer permeability. A) Transwell fitc/dextrans permeability assay data of multiple experimental repeats (n=6) of Wt HBEC or CARGFP HBEC monolayers either treated or untreated with TNF α (10ng/ml). B) Graph showing the percentage change in permeability between the Wt or CARGFP monolayers either treated or untreated at 30 or 60 minutes where there was a demonstrable change with the addition of TNFα (n=4).

4.2.3 Confocal imaging of junction markers in mixed populations of Wt HBEC and FLCAR HBEC

Following the data suggesting that the presence CAR at junctions is capable of maintaining their stability after the addition of TNF α it was necessary to determine whether there was alteration in other protein markers associated with both tight and adherens junctions. ZO-1 was used as a marker for tight junctions and was as expected seen to co-localise with CAR at epithelial junctions both in a resting state and in the presence of TNF α (figure 4.4a).

E-cadherin was used as a marker for adherens junctions. E-cadherin has previously been shown to be displaced from junctions that over-express CAR at junctions, specifically when the cytoplasmic tail of CAR is not phosphorylated at either the p-ser290/thr293 sites (Morton *et al*., 2013). As previously identified confocal imaging showed that when untreated there was little co-localisation of CAR and E-Cadherin to epithelial junctions. Following treatment with TNF α for 30 minutes the p-ser290/thr293 sites on CAR have been shown to be phosphorylated. The confocal imaging suggests that it also leads to an increased expression of E-Cadherin at epithelial junctions with a representative image showing increased red staining of E-Cadherin at junctions (figure 4.4b). This supports the previous assertion that where the p-ser290/thr293 sites on CAR are phosphorylated then E-Cadherin is able to be expressed at the junction.

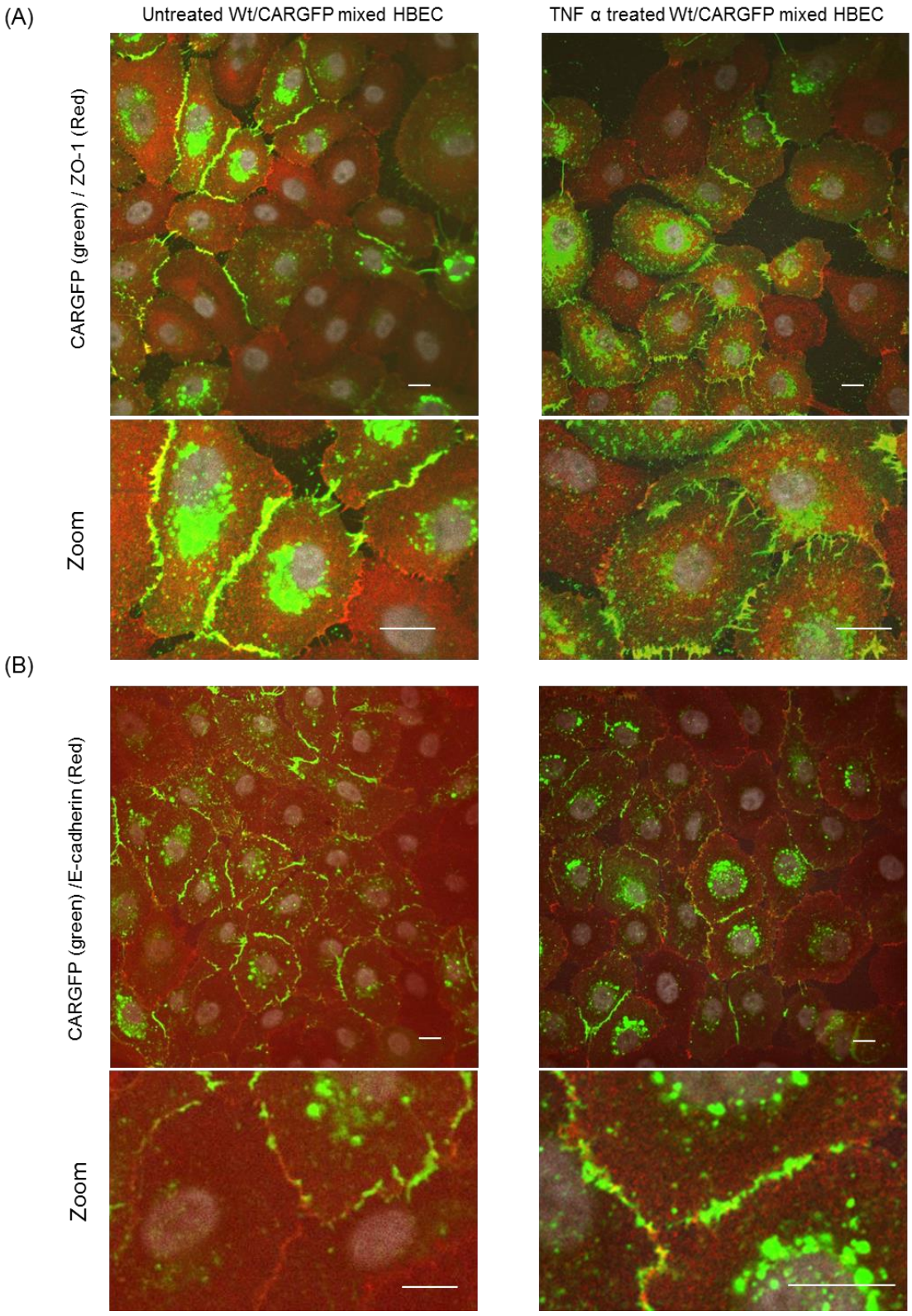


Figure 4-4: Confocal imaging showing maximum intensity projections of junction markers in HBEC following TNF α treatment: A) Tight junction staining (ZO-1 - red, CAR - green, co-localisation of both - yellow, nucleus - white) of HBEC +/- treatment with 10 µg TNF α for 30 minutes. B) Adherens junction staining (E-Cadherin – red, CAR - green, co-localisation of both - yellow, nucleus - white) of HBEC +/- treatment with 10 µg TNF α for 30 minutes. Scale bars are 10um.

4.2.4 Phospho-CAR alters leucocyte transmigration

The role of CAR at the cell membrane has already been established as more complex than simply homodimerising with adjacent epithelial cells. As described in the introduction, CAR is also able to act as a ligand for proteins on transmigrating leucocytes (Witherden *et al*., 2010, Zen *et al*., 2005) but the mechanisms underpinning this are unclear. In order to test the hypothesis that phosphorylation of CAR may be involved in leucocyte transmigration, a monocytic-derived cell line THP-1 (that express JAM-L) were incubated with control WT HBEC or CAR-GFP HBEC and allowed to undergo transmigration over 24 hours. In agreement with previous studies, data demonstrated that overexpression of CAR-GFP significantly increased THP-1 cell transmigration but not adhesion to the epithelial layer (Figure 4.5 a, b). Moreover, incubation with recombinant Ad5FK (Kirby *et al* 2000 inhibited THP-1 transmigration but had no effect on adhesion (Figure 4.5 a,b). This suggests that THP-1 migration might be dependent on the ability of CAR to homodimerise in *trans*. Alternatively, as the binding sites on CAR for JAM-L and Ad5FK overlap (Verdino *et al*., 2010, Witherden *et al*., 2010, Kirby *et al*., 2000), Ad5FK may inhibit THP-1 transmigration by competitively blocking THP-1 binding to CAR. Interestingly, THP-1 interaction with CAR-GFP HBEC led to a prolonged increase in p-ser290/thr293 CAR that peaked at around 4 hours post-THP-1 addition (Figure 4.5 c), which correlated with the time taken for THP-1 to fully integrate into HBEC monolayers.

To investigate whether this observed increase in phospho-CAR played a role in TEpM we analysed transmigration in previously described ser290/thr293 CAR phosphorylation site mutants DDCAR-GFP or AACAR-GFP as well as WT and CAR-GFP HBEC. Data demonstrated that expression of DDCAR-GFP but not

AACAR-GFP supported enhanced THP-1 transmigration across HBEC indicating that phosphorylation of CAR is required for this process (Figure 4.5 d). Confocal analysis further revealed that CAR-GFP and DDCAR-GFP but not AACAR-GFP were clustered around transmigrating THP-1 cells between HBEC cells (Figure 4.5 e). These data collectively demonstrate that CAR is required for efficient transmigration of leucocytes and this is promoted by phosphorylation of the CAR cytoplasmic tail.

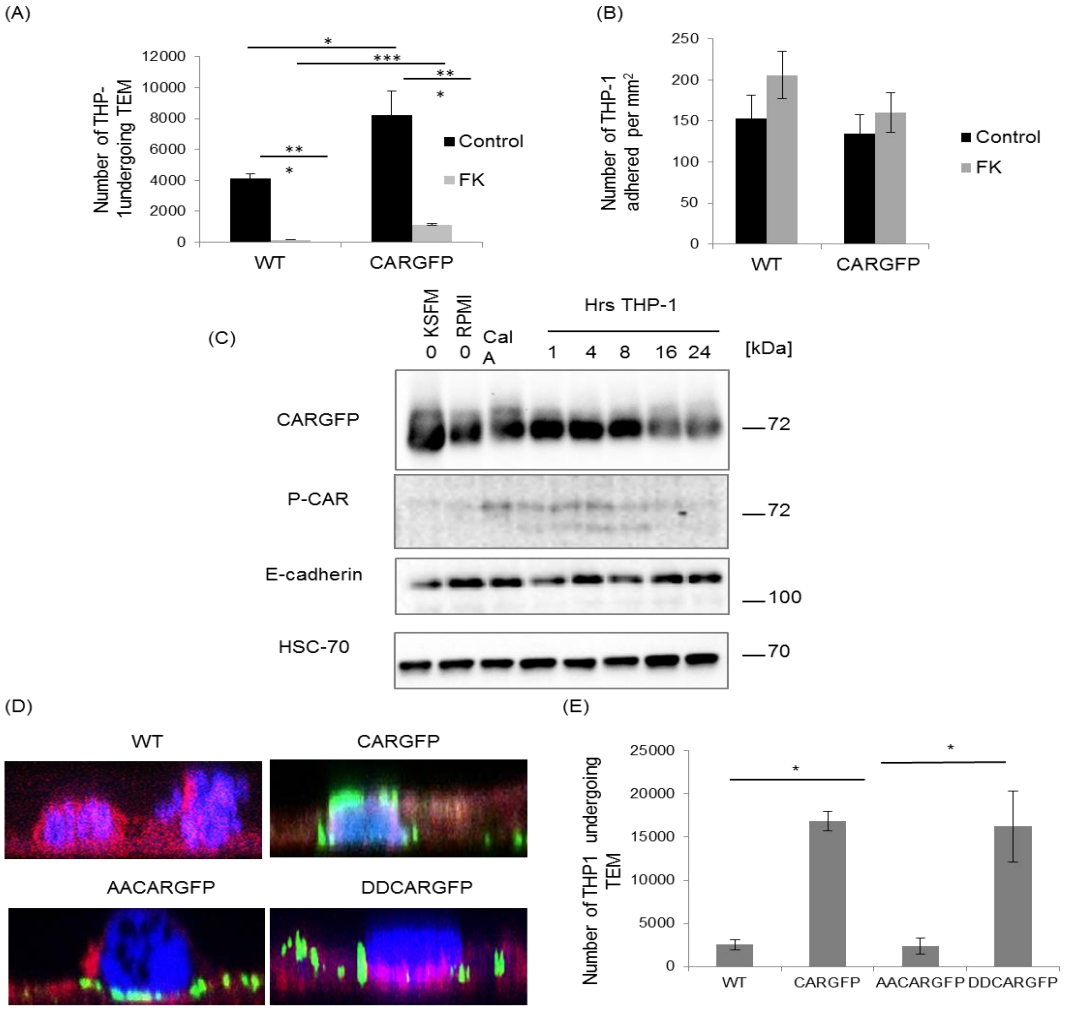


Figure 4-5 CAR phosphorylation does not affect adhesion but promotes THP-1 integration: (data from Dr. Penny Morton) (A) Epithelial transmigration of THP-1 cells was analysed using Transwell chambers. THP-1 cells were stained using cell-tracker orange before adding to the top well of transwell inserts with WT or CARGFP-HBEC monolayers grown on top and pre-treated with BSA control or recombinant Ad5FK. After 48hours cell-tracker orange stained cells in the bottom well were counted using FACS. (B) Quantification of THP-1 adhesion to wild-type or CAR-GFP HBEC cells. HBEC cells were grown to confluence in the presence of BSA control or recombinant Ad5FK before addition of THP-1 cells stained with cell-tracker orange for 24 hours. N=4 (C) Western blot of phosphorylated CAR during THP-1 transmigration. THP-1 were applied to CARGFP HBEC monolayers for the times indicated before lysis and western blotting using antibodies against p-CAR, GFP, E-cadherin and HSC-70 as a loading control. (D) Transmigration analysis as in 1B using wild-type, CARGFP, AACARGFP and DDCARGFP HBEC. (E) Example confocal images of THP-1 cells undergoing transepithelial migration. Cell tracker orange stained THP-1 cells were added to confluent monolayers of HBEC and fixed after 24 hours before confocal imaging to obtain z-stacks. Cell tracker orange THP-1 cells (blue), CARGFP (green) and actin (red) to show the position of the HBEC are shown. Z-slice shows the location of THP-1 in relation to HBEC monolayer and recruitment of CARGFP to THP-1. Error bars are SEM. *=p<0.05, **=p<0.01 ***=p<0.005.

4.2.5 TNF α induced CAR phosphorylation promotes TEpM of THP-1 cells

Given that phosphorylation of CAR can regulate THP-1 migration, and that TNF α promotes CAR phosphorylation, as we have shown, we next sought to clarify whether TNF α could promote integration of leucocytes within epithelial monolayers in a CAR-dependent manner. CAR-GFP HBEC monolayers stimulated with 10ng/ml TNF α showed significantly higher THP-1 integration after 4 hours than untreated monolayers (Fig 4.5 a). Moreover, WT HBEC (that express low levels of CAR) and AACAR-GFP HBEC did not exhibit an increase in THP-1 integration when treated with TNF α (Fig 4.5 a). These data show that phosphorylation of CAR is required for TNF α-induced transmigration of THP-1 cells. Importantly, although TNF α has been shown to promote TEM through increasing permeability of endothelial cells (Cain *et al*., 2010). The results from the permeability experiments show that CAR-GFP expression actually reduces TNF α-mediated paracellular permeability (figure 2.3b,c). This suggests that CAR does not promote TEpM via weakening of cell-cell contacts.

THP-1 have previously been shown to secrete TNF α (Satsu *et al*., 2006) so we next investigated whether this may act on the HBEC in a paracrine fashion to promote THP-1 transmigration. In support of this, pre-incubation of CAR-GFP HBEC with TNFR1 blocking antibodies resulted in reduced THP-1 integration into CAR-GFP HBEC monolayers (figure 4.5 b) there by confirming a requirement for TNFR1 in this process. Moreover, blocking TNFR1 inhibited both TNF α- and THP-1 induced CAR phosphorylation (figure 4.5 c, d) further demonstrating a requirement for TNF-TNFR1 engagement in controlling TNF α induced CAR phosphorylation. Taken together, these data show that TNF α secreted by THP-1 cells can induce CAR phosphorylation in epithelial cells via TNFR1 to promote THP-1 epithelial integration.

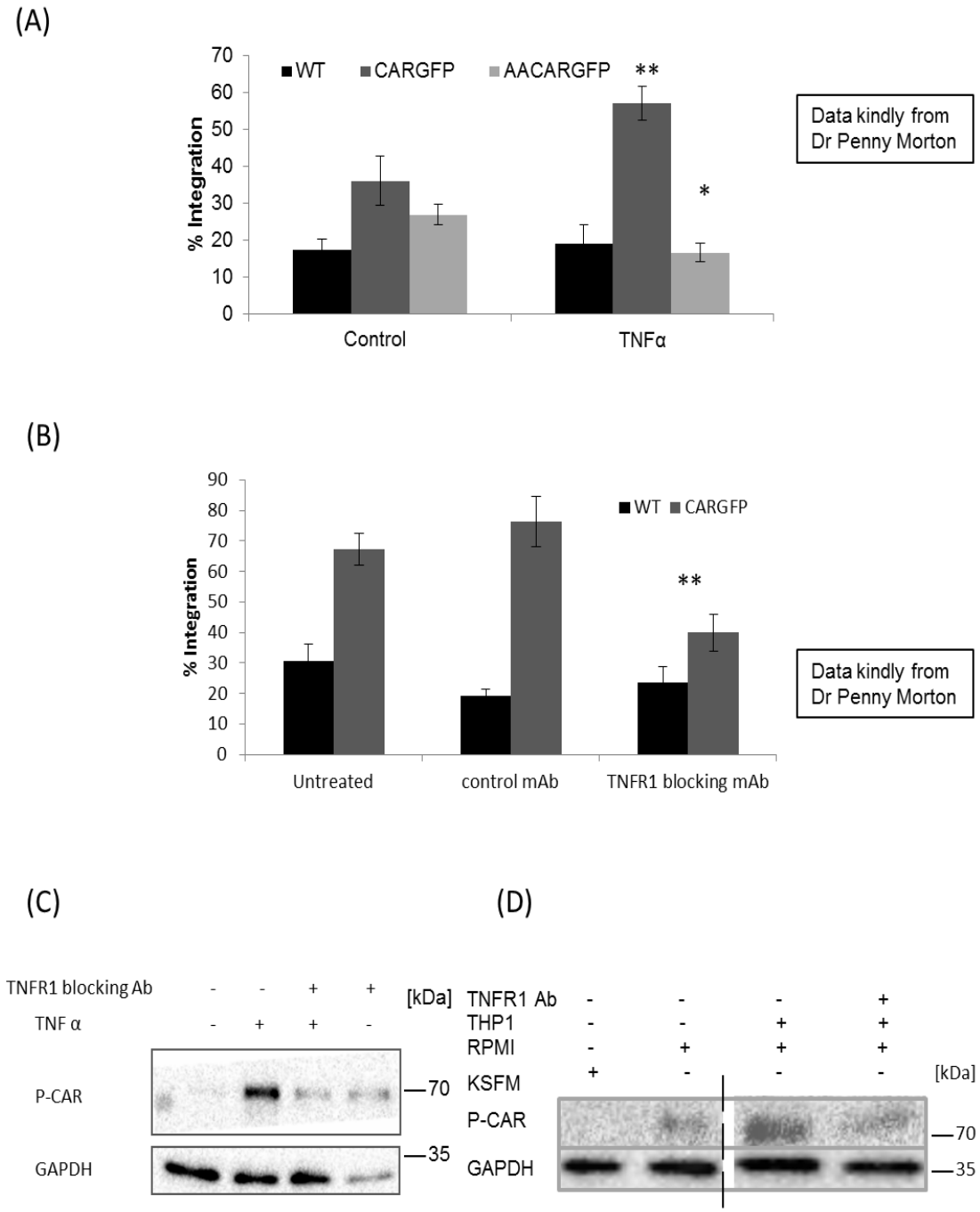


Figure 4-6: TNF α promotes trans-epithelial migration of THP-1 cells in a CAR dependent manner. (A) Analysis of THP-1 integration into WT, CARGFP or AACARGFP HBEC monolayers 4 hours after addition of THP-1. Where indicated, HBEC cultures were pre-treated with 10ng/ml TNF α for 1 hour prior to addition of THP-1. (B) Analysis of THP-1 integration into WT, CARGFP or AACARGFP HBEC monolayers 6 hours after addition of THP-1. Where indicated, HBEC cultures were pre-treated with 10µg/ml anti-TNFR1 blocking antibody or an isotype specific control antibody, for 1 hour prior to addition of THP-1. (C) Western blot analysis of phospho-CAR in CARGFP HBEC treated with 10ng/ml TNFR1 blocking antibody or a control antibody, and further treated with 10ng/ml TNF α where indicated. (N=3)(D) Western blot analysis of phospho-CAR in CARGFP HBEC cultured with THP-1 cells for 4 hours. Where indicated, CARGFP HBEC were treated with 10µg/ml TNFR1 blocking antibody prior to addition of THP-1 (N=3).

4.3 Discussion

In vitro data presented in this chapter provides a direct functional role for CAR in TEpM, as integration of THP-1 cells was reduced in the absence of CAR at the cell membrane. This process has previously been demonstrated in the TEpM of neutrophils through the heterophilic interaction between CAR on epithelial cells and JAM-L on neutrophils (Zen *et al*., 2005). THP-1 cells have also been shown to contain JAM-L and their transmigration through endothelial layers was impaired when JAM-L was blocked from interacting with its likely binding partner CAR (Guo *et al*., 2009). In the longer term this interaction is augmented by soluble JAM-L released by transmigrating neutrophils which extenuates the loss of barrier function through its binding to CAR which slows wound healing (Weber *et al*., 2014).

Data presented here shows that CAR is only active in mediating TEpM when it can be phosphorylated at the ser290/thr293 site. This phosphorylation event was a prerequisite for TNF driven TEpM. There have been several studies to show that the presence of TNF α leads to increasing epithelial permeability through a variety of methods. This firstly includes the internalisation of the apical tight junction, thereby causing disruption of cell-cell contacts and increased solute passage (Ivanov *et al*., 2004, Schwarz *et al*., 2007). TNF α has also been associated with the mechanisms that control cell survival and cell death, in particular via activation of NFκB (Saile *et al*., 2001) however the presence of CAR had no effect on signalling to MAPK or NFkB downstream of TNF suggesting that CAR does not play a role in these pathways. Therefore TNF α may also be causing an increase in permeability via the apoptosis of epithelial cells leading to holes forming in the epithelial raft. This process has been shown to be the case in HT-29/B6 intestinal cells with a doubling of their rate of death in the presence of

TNF α with a resulting fifty percent loss in their TER (Gitter *et al*., 2000a, Gitter *et al*., 2000b). This response is not universal, however, with multiple other studies showing the epithelium is highly conserved during cell apoptosis with cells stretching to fill any spaces caused by cell death and thereby preserving the barrier function (Madara, 1990, Madara, 1989, Moore *et al*., 1989, Florian *et al*., 2002). Importantly all of these events are shown to occur over a 24 hour period where as the effect on CAR at cell junctions occurs much more rapidly with phosphorylation within fifteen minutes.

Significantly, for the TEpM data, the increased permeability effect was not found to be the case in the presence of CAR, which suggests that the changing movement of leucocytes is not simply due to the physical loss of cell contacts. From the data shown this may reflect the importance of p-ser290/thr293 CAR at stabilising the junction but could it also be related to the alteration of other tight junction proteins in the presence of CAR. Occludin but not claudin-4, ZO-1 or E-cadherin for example was shown to be lost from junctions in response to TNF and IL18 in Human colonic epithelial (Caco-2) cells (Marchiando *et al*., 2010, Lapointe and Buret, 2012). Lapointe and Bur*et al*so noted that in Caco-2 cells there was no rise in permeability but there was an increase in TEpM with this occludin loss. They suggest that fine changes in epithelial barrier structure, although too subtle to alter paracellular permeability, may be of great physiological significance by accommodating leukocyte transmigration.

These subtle changes with the loss of tight junction proteins does not account for the need for CAR to be present at the epithelial membrane and capable of phosphorylation for TEpM to occur. Neutrophils are known to secrete TNF α when in contact with endothelial cells to increase vascular leakage (Finsterbusch *et al*., 2014, Satsu *et al*., 2006). This TNF α secretion response has implications

for the results seen in this chapter. THP-1 cells alone were capable of driving CAR phosphorylation but this effect was lost in the presence of a TNFR1 blocking antibody implying a similar paracrine release of TNF from THP-1 was sufficient to drive p-ser290/thr293 CAR. It was this phosphorylation effect that was shown to be necessary for THP-1 trans-migration demonstrating that CAR plays an active role in this second step in TEpM. However, this process was not associated with leakage of solute through the epithelial barrier as the presence of CAR instead reduced the expected increase in permeability. Rather than as Lapointe and Buret suggest the loss of tight junction proteins may lead to subtle structural changes enabling TEpM CAR may instead be playing a role in maintaining junction integrity so that it is still present to act as a ligand for the migration of leucocytes attracted to a site of inflammation (Lapointe and Buret, 2012).

Two alternative JAM's, endothelial cell-selective adhesion molecule (ESAM) and JAM-C in contrast have been shown to increase junctional permeability when compared to knockout mice in endothelial layers rather than the epithelium (Luissint *et al*., 2014). This effect is associated with myosin light chain phosphorylation leading to actin contraction (Orlova and Chavakis, 2007) and therefore may explain the difference seen with CAR as its presence in inflammatory conditions appears to stabilise actin at the cell junctions rather than promote its contraction. This is an interesting finding as tight junction proteins along with the adherens junctions, are intimately linked to the peri-junctional acto-myosin ring, a belt like structure formed by actin and myosin II that encircles the apical pole of epithelial cells. This belt projects actin filaments that interface with the tight junction and thus circumferential contractions of the peri-junctional actomyosin ring regulate tight junction structure and para-cellular permeability (Ulluwishewa *et al*., 2011). It also ties into previous work that has shown that

where CAR in epithelial cells is phosphorylated E-cadherin is mobilised to cell junctions which would aid junction stability and reduce permeability (Morton *et al*., 2013). Although the phosphorylation of tight junction proteins does not always lead to reduced permeability as seen with ZO-1 at epithelial junctions are associated with a reduction in TEER (Stevenson *et al*., 1989) other JAM family members, JAM-A and JAM-2, have also been found to stabilise cell junctions. This was seen between endothelial and epithelial cells respectively and led to reduced permeability when they were phosphorylated at serine sites (Ebnet *et al*., 2003, Iden *et al*., 2012). Therefore the increased TEpM seen with p-ser290/thr293 CAR must be related to its interaction with the THP-1 cells rather than the general porousness of the epithelial layer. Data shown in the previous chapter also suggests that this is not a reflection of downstream signalling events as there was no alteration in the pathways associated with TNF α signalling when p-ser290/thr293 CAR occurred. Therefore the TEpM response to TNF α driven p-ser290/thr293 CAR may reflect an alteration in the binding of CAR to JAM-L to aid leucocyte binding and movement. The extracellular domain of JAM-A for example will bind significantly more strongly if it can occur *in cis* rather than *in trans* (Monteiro *et al*., 2014). Therefore if the cytoplasmic phosphorylation of CAR is having an effect on the extracellular domain then it could be mediating the impact on TEpM.

The paracrine release of TNF α from THP-1 during adhesion and the resulting CAR phosphorylation driving THP-1 migration also suggests a possible reason for the conflicting results for anti-TNF treatments in inflammatory conditions in the lungs (Erin *et al*., 2006, Brightling *et al*., 2002, Rennard *et al*., 2007). These studies have only ever shown a limited response to systemic anti-TNF therapies in particular in asthma which may reflect that the movement of inflammatory cells

that drive the disease can be released by the cells themselves and act over very short distances by controlling tight junction proteins such as CAR that are found in the adjacent epithelial cells.

In conclusion, these results support a novel mechanism where CAR can control TEpM of leucocytes through its own post-translational modification in response to a TNF α inflammatory stimulus. These findings implicate CAR as an important mediator of immune cell recruitment to sites of epithelial inflammation.

5 Inflammation leads to CAR phosphorylation and immune cell migration *in vivo*

5.1 Introduction

The conclusions drawn from the last two chapters have been based on the use of immortalised cell lines. The understanding of the importance of CAR in inflammation is limited and therefore many of the fundamental principles underlying any role it may play are unknown. *In vitro* models are essential in the development of any understanding of these principles and have been widely used previously in the study of epithelial junctional dynamics in asthma (Xiao et al., 2011, Hackett et al., 2011). These cells were used as they allowed for stable expression of CAR in a controlled and reproducible form. In so doing the fundamental biology of CAR could be interrogated. Primary cells in *in vitro* models provide a closer reflection of living systems as they have not been manipulated in such a way as to ensure immortality. However, they have been shown to exhibit a high degree of variability between donors, experiments, and passage, particularly with respect to development of epithelial junctional stability (Stewart et al., 2012). Primary cells are also costly and therefore unsuitable for large scale experiments, have a finite lifespan and can be difficult to manipulate as required by these experiments to alter the formation of the CAR cytoplasmic tail. Immortalised cell lines therefore represent an attractive alternative to investigate basic biological principles.

Nevertheless, there remains concern that immortalised cells do not replicate living systems. Animal models have been established as providing additional relevant information for our understanding of inflammatory conditions in the complex interactions that occur in the human lung (Vargaftig, 1999, Hraiech et

al., 2015, Erle and Sheppard, 2014). This chapter therefore addresses the physiological relevance of CAR in respiratory inflammation by testing the conclusions of the preceding two sections in animal models.

5.2 Results

5.2.1 Mouse lung response to acute inflammatory stimulation

The initial experiments investigating a role for CAR phosphorylation in leucocyte transmigration in response to inflammatory conditions were all performed *In-vitro* but suggest at a physiologically relevant response. Following the finding that the CAR phosphorylation response *in vitro* occurs rapidly in response to TNF α a mouse model was chosen to reflect an acute inflammatory response. The inflammatory conditions were provided by intranasal inhalation of TNF α 24 hours prior to animal sacrifice. This challenge led to a significant response in the mouse lung with a rise in the number of neutrophils seen in the peri-bronchial region (figure 5.1) and bronchoalveolar lavage (BAL) fluid (figure 5.2).

Importantly this increase in neutrophil egression measured in bronchoalveolar lavage and by H&E staining in the same animals corresponded with a dramatic increase in CAR phosphorylation at junctions between small airway epithelial cells as detected by immunohistochemical staining of fixed lung tissue (figure 5.3 a). This was not due to increased presence of total CAR at tight junctions in response to TNF α stimulation, as equivalent amounts of CAR was seen at junctions in both the PBS control mice and the TNF stimulation group (figure 5.3 b). These data are therefore suggestive that CAR, which is present at tight junctions during resting conditions in the lung, is being phosphorylated in inflammatory conditions.

Further analysis of lung tissue revealed that 24 hours following TNF α stimulation there was no clear change in localisation of other epithelial junctional markers including P120, ZO-1 and E-cadherin (figure 5.4 a,b,c). This would suggest that the epithelium remains intact and the change in neutrophil number seen in the BAL data is not a reflection of the loss of the epithelial barrier effect but instead an active process that allows for leucocyte egression.

These data show that CAR phosphorylation and neutrophil recruitment into the broncho-alveolar spaces occur at similar time-frames and similar locations in the lung suggesting that exogenously applied TNF α stimulates CAR phosphorylation and promotes migration of leucocytes in the bronchial lumen.

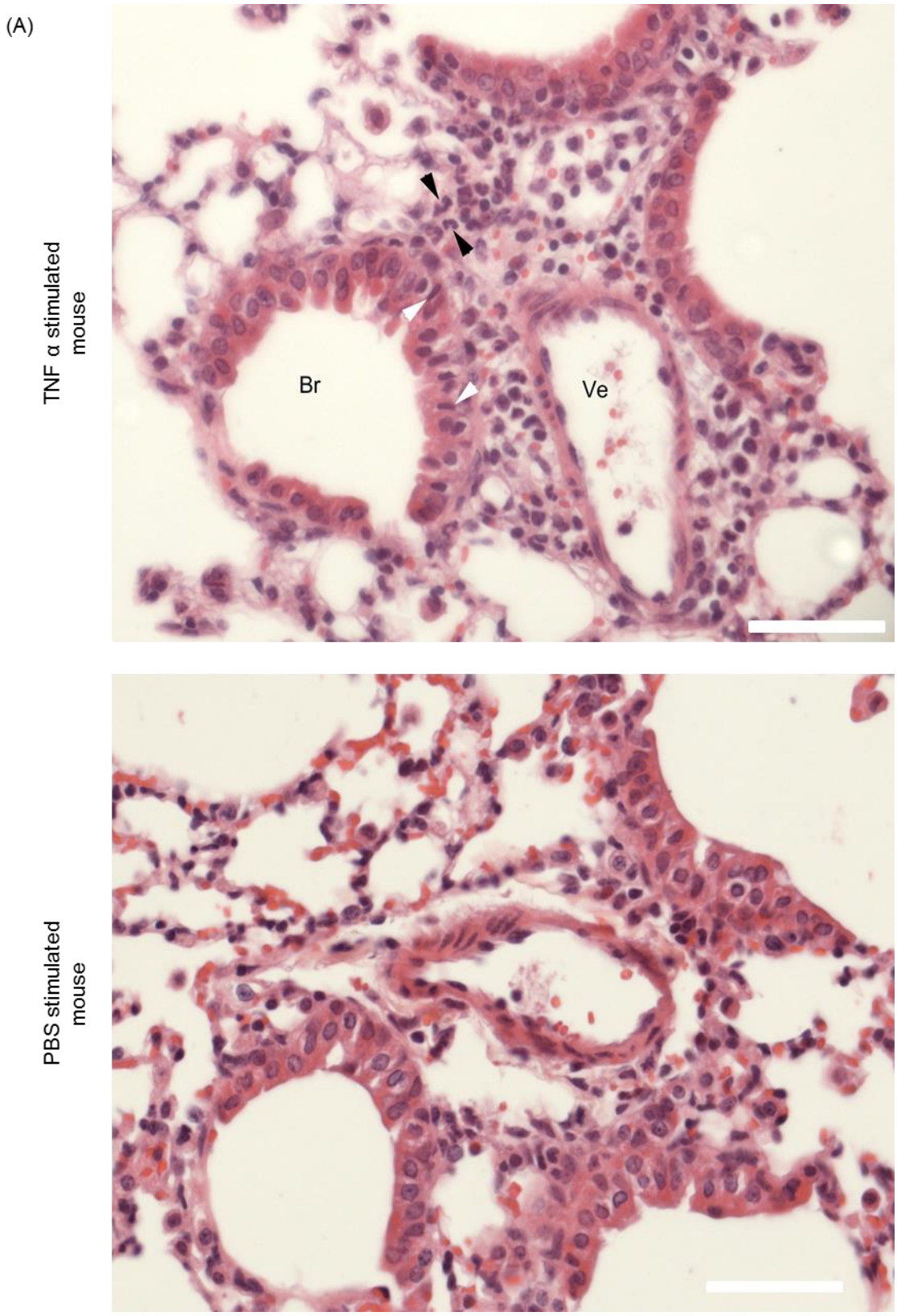


Figure 5-1 Acute inflammatory mouse model with increased neutrophil presence in response to TNF stimulation. A) Mouse lung sections of either TNF or PBS stimulated animals. Neutrophils marked with black arrows in the tissue space and leukocytes migrating through the epithelial layer marked by white arrows. Scale bars are 50μm.

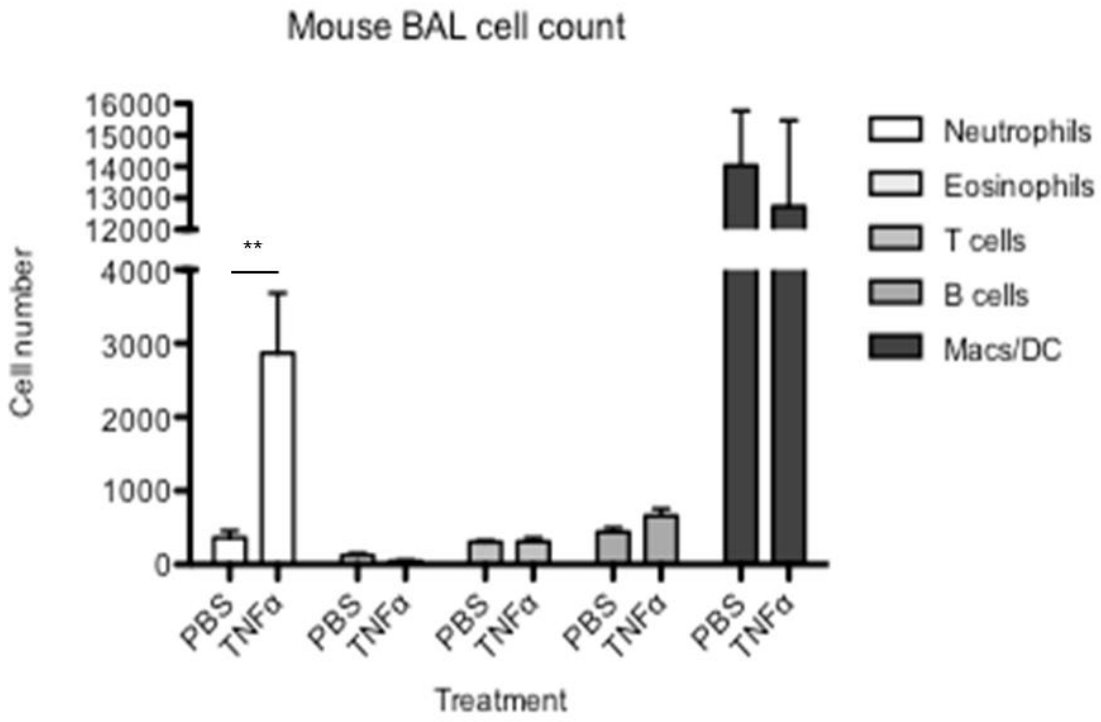


Figure 5-2: Acute inflammatory mouse model with increased neutrophil presence in response to TNF stimulation. Flow cytometry results from the bronchiolar lavage results of the pooled data from the control and treatment groups (n=6 per group). Error bars are SEM and ** =p0.009

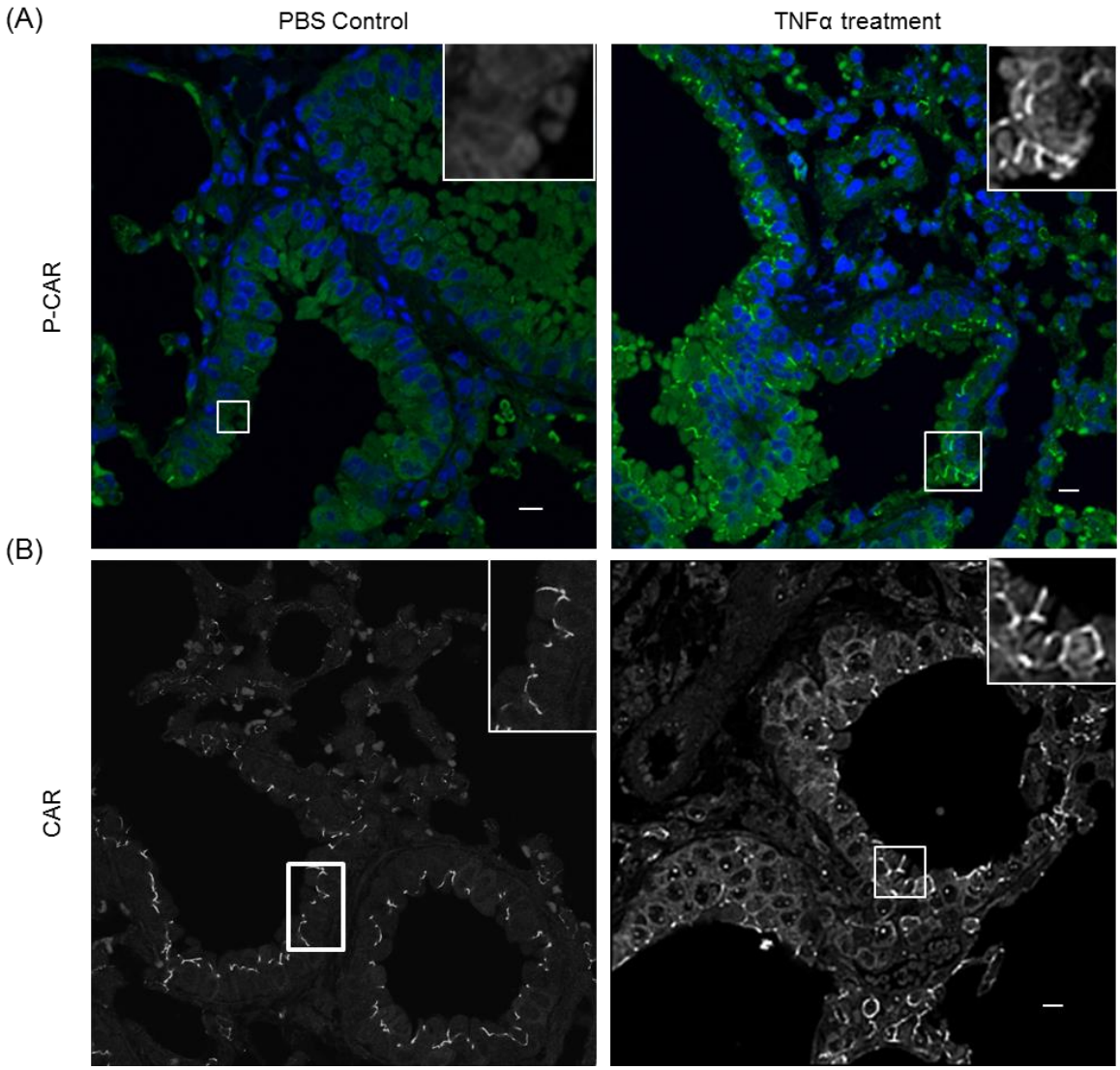


Figure 5-3: Mouse lung model of acute TNF α stimulation leads to CAR phosphorylation. A) p-ser290/thr293 CAR staining (green) of mouse lung tissue following TNF stimulation or PBS control. Nuclei are shown in blue. Zoomed images of individual cells shown in each corner B) CAR staining of the same mouse lung tissue following TNF or PBS treatment showing no variation in CAR expression. Images representative of 6 mouse lungs. Scale bars represent 10μm.

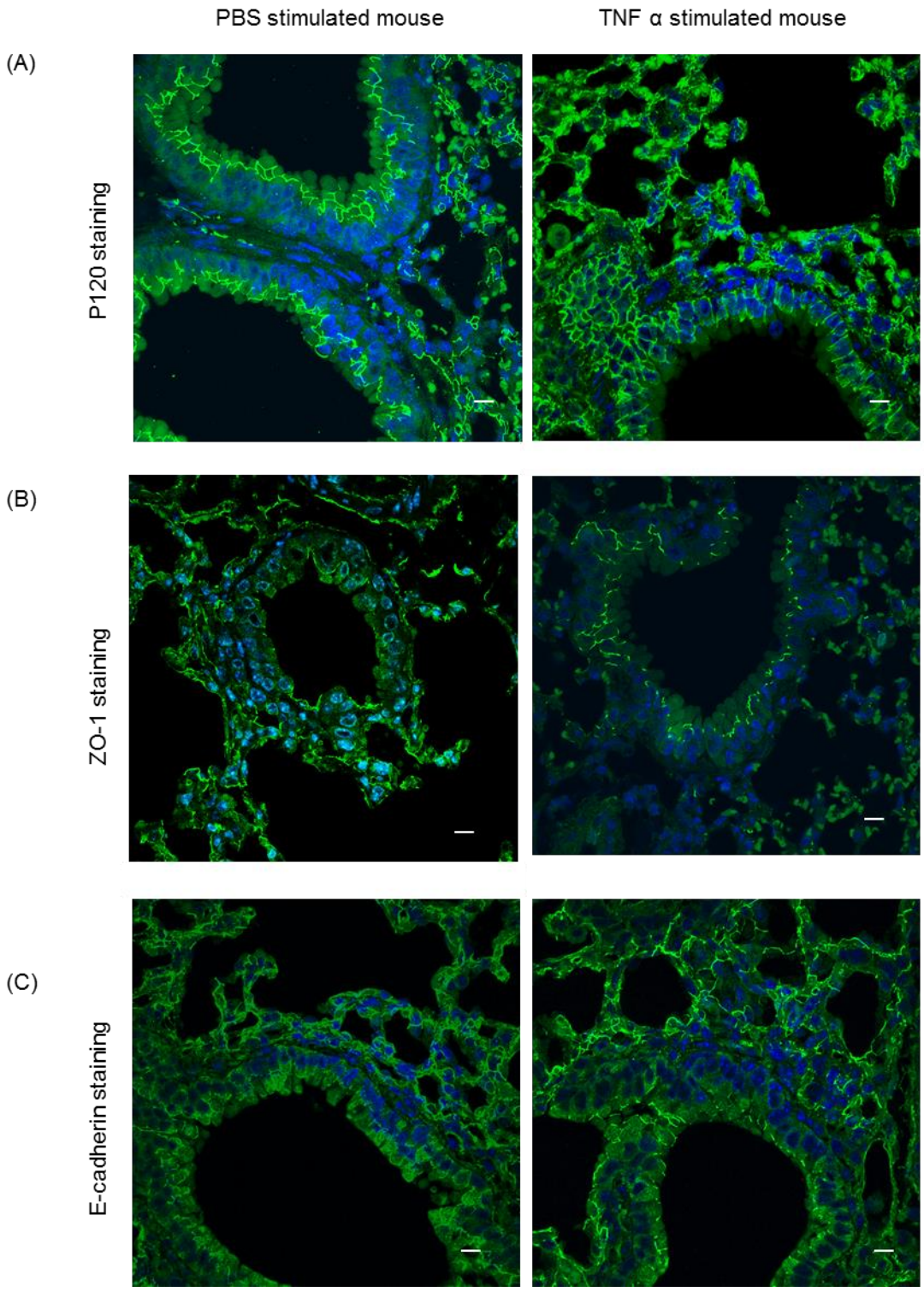


Figure 5-4: Junction marker staining is unaltered in an acute inflammatory mouse lung model. A) P120 staining. B) ZO-1 staining. C) E-cadherin staining. All antibodies in green, blue are DAPI staining (nuclei). Images representative of 6 mouse lungs. Scale bars represent 10μm.

5.2.2 Mouse lung response to TNF α in the presence of Ad5FK

The initial *in vivo* data demonstrated a correlation between the phosphorylation of CAR and increased transmigration of neutrophils in response to TNF α stimulation but did not provide a causal link between the two. To determine whether this is the case, the experiments were repeated but with the additional inclusion of mice pre-treated with intra-nasal inhalation with Ad5FK. Ad5FK was used as it had been shown to both block p-ser290/thr293 CAR (figure 3.13) and CAR mediated THP-1 migration (figure 4.5 a). Analysis of immune cell infiltration revealed that the presence of Ad5FK resulted in a significant reduction in the number of neutrophils found in the BAL (Figure 5.5). This implies that blocking CAR homodimerisation and phosphorylation leads to reduced leucocyte transmigration through the epithelial layer and into the airways in response to TNF α stimulation.

Interestingly the *in vitro* experiments used the THP-1 cell line which was derived from the blood of a patient with acute monocytic leukaemia. These cells therefore are used to model monocyte or macrophage responses *in vitro*. As a cell line THP-1 cells are useful as they have a homogenous genetic history thereby ensuring a reproducible response. They, however, do have some differences in their response when compared to primary monocyte or macrophage cells, including the effect of the cytokines such as IL 10 and IL 27 (Qin, 2012). This could be the cause for the difference shown with the *in vivo* data which shows that there is a stronger response from neutrophils rather than macrophages.

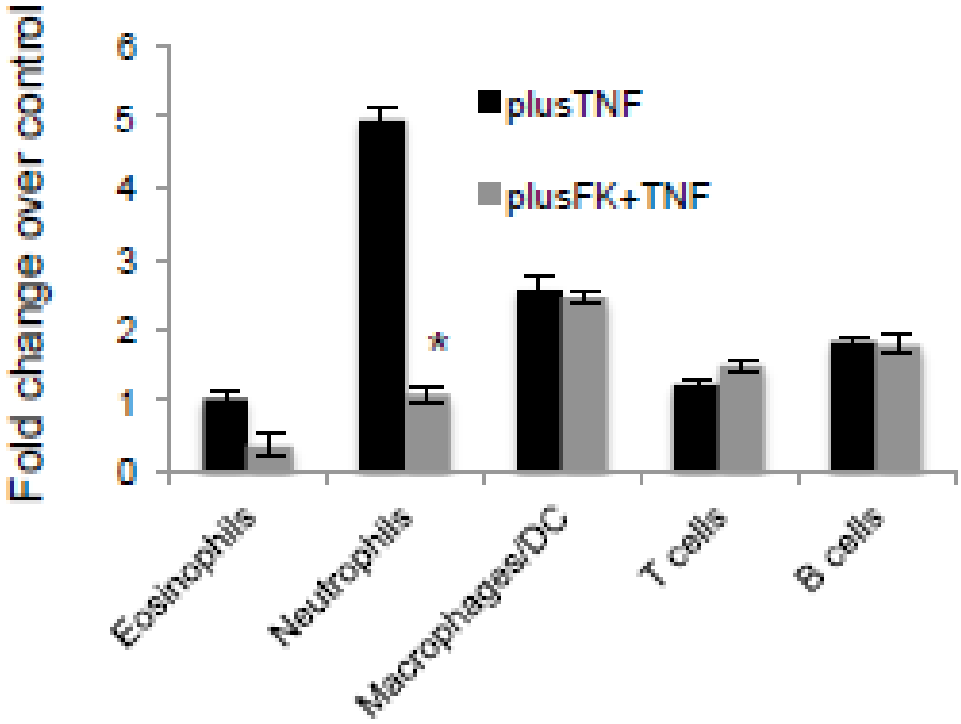


Figure 5-5 Acute inflammatory mouse model with loss of increased neutrophil presence in response to TNF stimulation with the addition of Ad5FK. Flow cytometry results from the bronchiolar lavage results of the pooled data from the control and treatment groups (n=4 per group). Error bars are SEM and * = p0.029

5.2.3 Mouse lung response to chronic inflammatory stimulation

To further evaluate the effect of CAR activity on lung inflammation an alternative mouse model was used to reproduce chronic inflammatory conditions. The Ovalbumin challenge model of chronic lung inflammation was used. Although it does not exclusively drive TNF α stimulation it is well established as providing a reflection of many of the findings seen in asthmatic conditions in the lung (Kumar *et al*., 2008, Nials and Uddin, 2008). These experiments were carried out in the lab of Dr Gisli Jenkins (University of Nottingham) and fixed tissues and protein samples provided to us for analysis.

H&E staining of the mouse lung tissue demonstrates significant architectural changes in response to the ovalbumin challenge (figure 5.6 a,b). This response is driven by extensive peri-vascular and peri-bronchiolar leucocyte infiltration (figure 5.7 a,b) reflecting the strong inflammatory response generated by this model. The leucocytes seen were predominantly macrophages and neutrophils as highlighted in figure 5.7b.

Further immunostaining revealed that, as seen with the acute inflammatory model, a strong CAR p-ser290/thr293 increase was observed at the cell membrane of epithelial cells associated with an influx of inflammatory cells (figure 5.7).

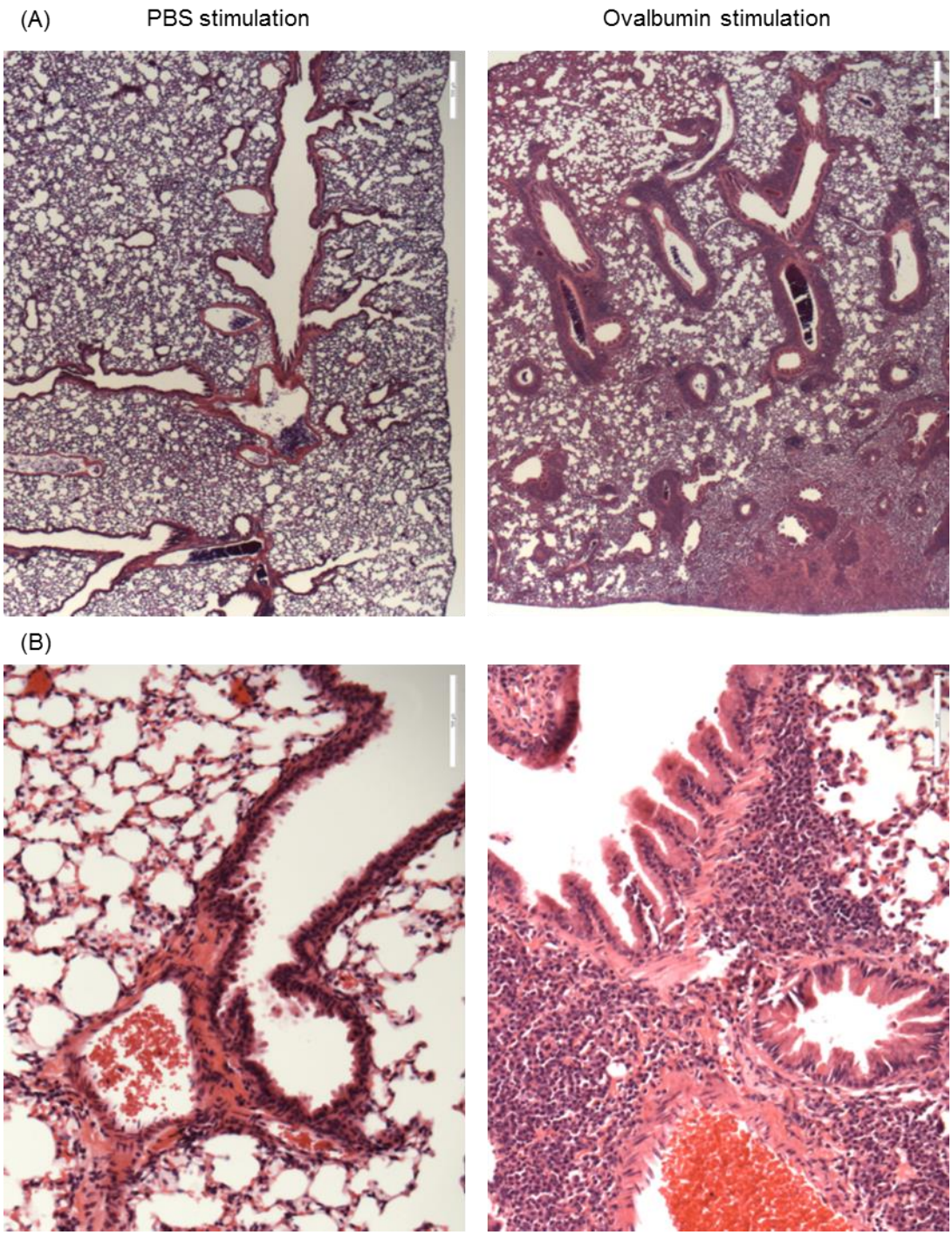


Figure 5-6: H&E staining of ovalbumin treated mouse lung. A) H&E staining of widefield view of PBS and ovalbumin mouse lungs. B) H&E stained images focused on small airways. Images representative of 6 mouse lungs.

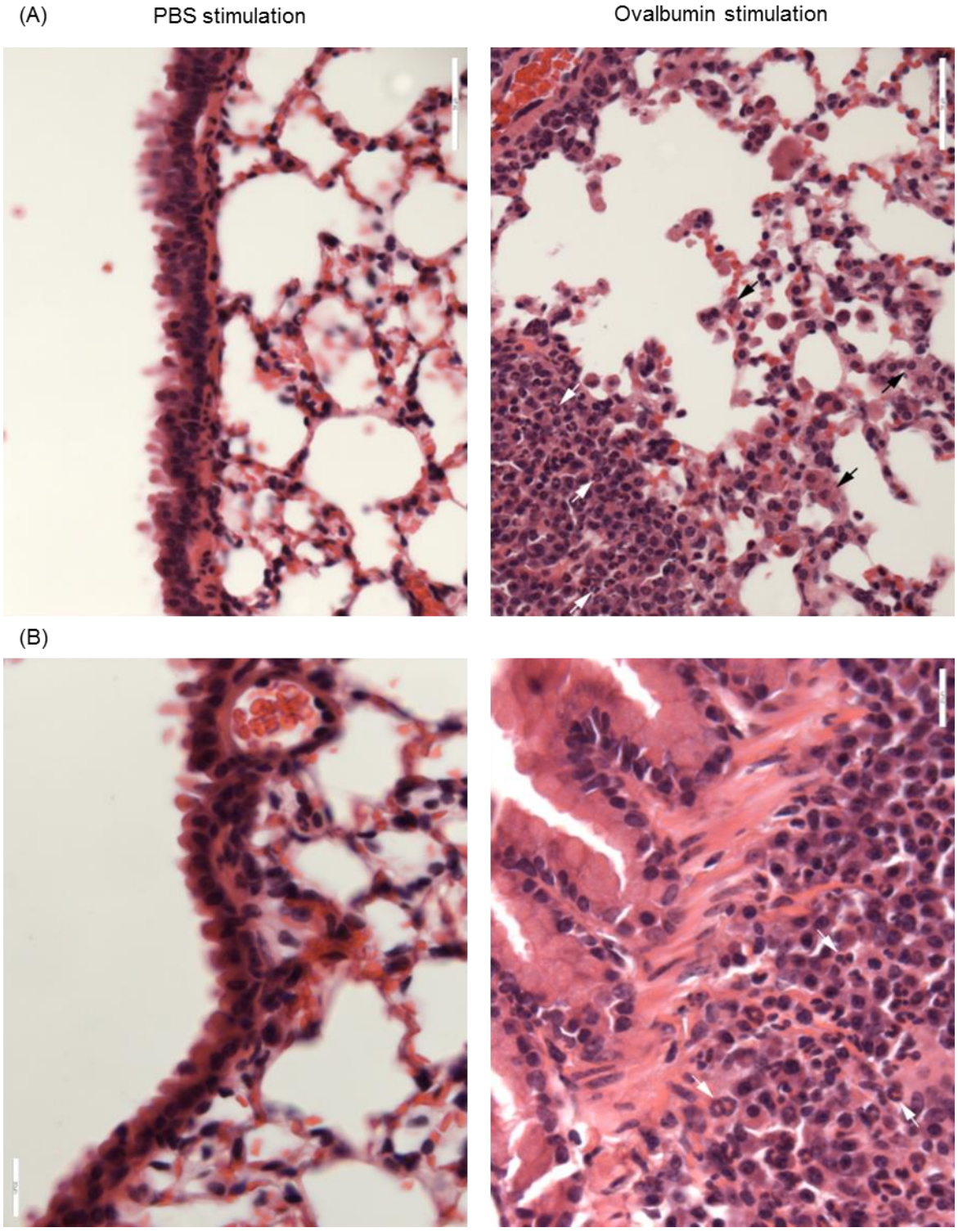


Figure 5-7: H&E staining of ovalbumin treated mouse lung (Higher magnification). A,B) H&E stained images focused on leukocyte cell types in the sub epithelial zone in mouse lung tissue. Representative neutrophils highlighted with white arrows and macrophages with black arrows. Images representative of 6 mouse lungs.

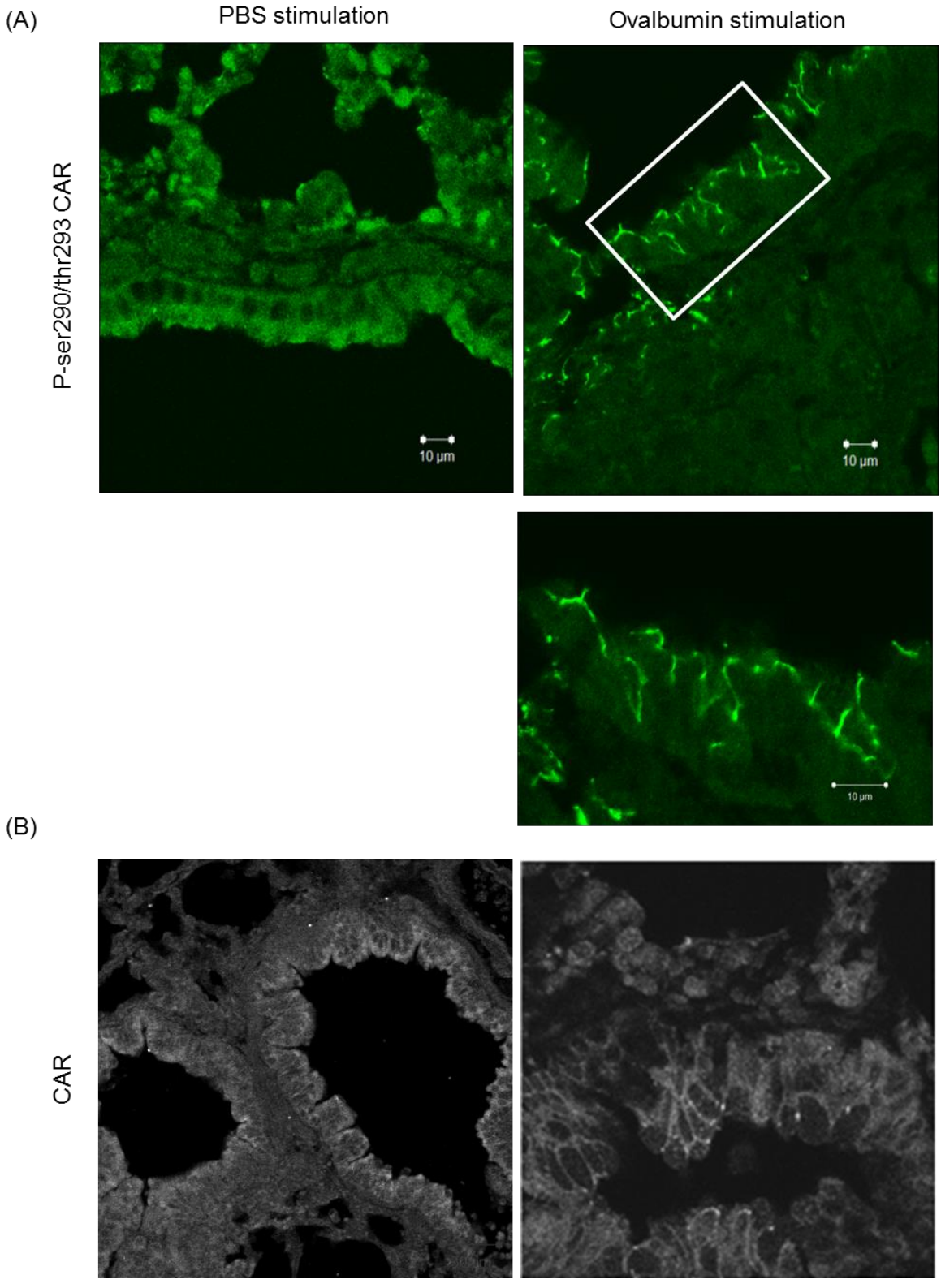


Figure 5-8: Confocal imaging of CAR phosphorylation of mouse epithelium in response to ova stimulation. A) Imaging for p-ser290/thr293 CAR in ovalbumin treated mouse lung with a zoomed image of epithelial junctions. B) Confocal imaging of CAR in ova albumin treated mice. Images representative of 6 mouse lungs.

5.3 Discussion

This thesis set out to determine whether CAR phosphorylation in response to TNF α is of physiological relevance through CAR's known interaction with leucocytes during TEpM.

The *in vivo* models used mice as the animal system which does lead to limitations in its possible application in human biology. In particular mice do not naturally suffer with inflammatory conditions such as asthma and as such model systems looking to recreate similar inflammatory lung states require artificial sensitisation. Therefore an alternative tissue engineered model using human epithelial cells in combination with other parenchymal cells to develop artificial human lung equivalents may provide additional relevant data (Bucchieri *et al*., 2012). However, the two mouse models used here both show that CAR is phosphorylated at cell membranes during inflammatory conditions. The fact both mouse strains demonstrate a CAR phosphorylation response implies a consistent physiologically relevant response which is important given previous reports that the genetic background of the mouse strain can significantly affect the presence and function of specific junctional proteins (Schenkel *et al*., 2004, Woodfin *et al*., 2009).

It is also interesting to note that the phosphorylation effect is maintained despite chronic inflammatory stimulation. Vincent *et al.* have previously shown that CAR mRNA levels in vascular endothelial cells are reduced following prolonged exposure to both TNF α and IFN γ but not immortalised A549 respiratory epithelial cells (Vincent *et al*., 2004). Therefore the suggested feedback mechanism in response to inflammation caused by the loss of CAR in endothelial cells does not appear to occur in the mouse lung epithelium as CAR was still

present and phosphorylated at the cell membranes despite prolonged inflammation. This consequently indicates that CAR may play a role in chronic inflammatory conditions in the lung and more specifically this role is relevant to the epithelium rather than the endothelium as there is no down regulation of its expression in these cells.

The significance of CAR phosphorylation in response to TNF α is difficult to prove *In vivo* as the mouse model by its very nature is a complex system. Confirming a direct link between CAR phosphorylation and TEpM would be greatly enhanced by studying this in a CAR knockout model. This in itself would be challenging as a CAR knockout is a lethal mutation by day 12 in mouse embryonic development (Chen *et al*., 2006). Therefore a lung conditional knockout mouse using either a Cre/loxP or Flp/FRT system would be required to ensure a spatial and temporal specific effect (Hall *et al*., 2009). Data here shows a correlation between increased neutrophil migration and CAR phosphorylation and combined with our *in vitro* data suggests a link between these two events. The *in vivo* neutrophil response to TNF α is reduced in the presence of Ad5FK showing that CAR is required for TNF α stimulated egression in the lung and suggests that CAR homerdimerisation at the cell membrane is required. This supports a role for CAR phosphorylation as this was shown to be blocked in the presence of Ad5FK. However since Ad5FK binds CAR on the same domain as JAM-L this result may also reflect competitive binding preventing the interaction of leukocyte proteins with CAR.

6 Discussion

6.1 CAR phosphorylation in inflammatory conditions

In this thesis I have set out to determine whether there is a physiological relevance for the two previously published, and one putative phosphorylation sites on the cytoplasmic tail of CAR during the inflammatory response in lung epithelial cells. Threonine290 and serine293 sites can be phosphorylated in response to inhibition of phosphatases (Morton *et al*., 2013) and we additionally identified a putative phosphorylation site at tyr269. Phosphorylation of the threonine290/serine293 sites was shown to control stability of E-Cadherin at the cell membrane (Morton *et al*., 2013). This finding suggested a possible function for these phosphorylation sites during both the normal inflammatory response and in disease as once at the cell membrane E-cadherin would be able to stabilise epithelial junctions and maintain its barrier function. This is of particular relevance to inflammatory conditions in the lung such as asthma as previous work has shown a down regulation of junctional proteins including E-Cadherin (Xiao *et al*., 2011). Therefore, phosphorylation of these sites during inflammation would indicate a potentially important role for CAR in the inflammatory processes. However, the importance in the overall function of CAR within the cell, and interaction with other cells, was not been clearly established.

Data presented in this thesis is the first to demonstrate that CAR phosphorylation can be triggered by an inflammatory stimulus, in particular in response to the pro-inflammatory cytokines TNF α and IL-5. TNF α is a pleotropic cytokine with multiple effects on almost all differentiated cells including the epithelium. In particular TNF α has been shown to have multiple effects on tight junction proteins in both epithelial and endothelial cells including causing the

internalisation of proteins such as JAM-1, occludin, and claudin-1 and 4 (Al-Sadi *et al*., 2009, Feng and Teitelbaum, 2013), which contributes to control of junction stability in these cells. This suggests that CAR phosphorylation downstream of TNF may play a role in the inflammatory cascade, possibly through control of E-cadherin and junction integrity. As outlined in the introduction this is also of particularly relevance in the respiratory epithelium as those patients with severe steroid resistant asthma can have their airway hyper-responsiveness controlled by TNF α inhibition with etanercept (Holgate, 2010, Morjaria *et al*., 2008, Howarth *et al*., 2005, Berry *et al*., 2006). This effect is not universal, though, (Erin *et al*., 2006, Brightling *et al*., 2002) and may reflect the time dependent effect seen in the *in-vitro* data where phosphorylation in response to cytokines was lost after a few hours. This transient phosphorylation event implies a method of auto regulation to prevent an uncontrolled inflammatory response as it is self-limiting. However, CAR phosphorylation was still evident in our chronic inflammation model suggesting that this autoregulation does not happen in cases of chronic stimulation of airway inflammation. This challenges previous work that found alternative junctional proteins, including ZO-1, occludin and E-cadherin were downregulated in patients with asthma (de Boer *et al*., 2008, Xiao *et al*., 2011). This may reflect the difference in the model system used which relied on recurrent stimulation of normal tissue compared to the intrinsically abnormal cells seen in asthmatic airways. Previous work has shown down regulation of CAR expression following chronic exposure to TNF α and Inf γ (Vincent *et al*., 2004). However, these observations were not reproduced when the same experiments were performed in respiratory cells (Vincent *et al*., 2004). Therefore there may be a specific response seen in the lung epithelium that enables CAR

phosphorylation to be maintained in chronic inflammatory conditions such asthma.

This contrasts with IL-5, the other cytokine shown to cause phosphorylation of the same sites on CAR. IL-5 is associated with TH2 derived inflammatory responses and in particular acts as a chemo-attractant to eosinophils. The results shown here confirm previous reports which showed that IL-5 activates PKCδ activity thereby suggesting that IL-5 could use a similar pathway to TNF to drive phosphorylation of the cytoplasmic tail of CAR (Bankers-Fulbright *et al*., 2001). Its activation of PKCδ could also point to a possible synergistic effect with TNFα as both cytokines would be driving the same pathway. IL-5, however, has not previously been shown to have any effect on epithelial cells (Al-Sadi *et al*., 2009). The IL-5 receptor has never been identified on epithelial cells and therefore the physiological relevance of IL-5 in the phosphorylation of CAR may be limited. IL-5 does act as a chemoattractant to eosinophils in particular therefore its phosphorylation effect may be of more relevance to endothelial cells to aid eosinophilic extravasation and accumulation in tissues. In this position a synergistic effect with the pro-inflammatory TNF α could lead to a rapid extravasation of leukocytes. The work in this thesis did not extend to the endothelium as the focus was the role that CAR can play in the complex function of the respiratory epithelium. However, further work could concentrate on its function within endothelial cells, particularly with reference to determining whether there is an additive effect of using both IL 5 and TNFα together.

Several alternative cytokines were also tested to determine whether they also drove the phosphorylation of CAR at the serine/threonine sites. As shown none of these other cytokines caused CAR phosphorylation despite their well-established role during inflammation of the lung epithelium, in particular in patients with

asthma. IFN γ and IL-17 have also been shown to activate PI-3K showing that activating PI3K is not sufficient to drive CAR phosphorylation (McKay *et al*., 2007, Prasad *et al*., 2005). The fact that TNF controls CAR phosphorylation in these conditions suggests that the activation of CAR occurs in a targeted and therefore controllable and adjustable manner. The lack of response to these cytokines may also reflect a difference in organ specificity. Section one highlighted the difference seen in organ expression of CAR. The response from PI-3K to IFN γ was seen in intestinal cells which are known to express CAR and which may respond differently to cytokines than airway epithelial cells (McKay *et al*., 2007). As yet CAR phosphorylation in the intestine has not been investigated and would be an interesting avenue to pursue further. It also supports the proposed pathway requiring the stimulation of both PI-3K and PKCδ. Theoretically the two processes could act independently to drive CAR phosphorylation. The previously reported PI-3K response to IFNγ and IL-17 would imply that they should be able to drive CAR phosphorylation if this kinase alone was required. Given that they do not have this ability therefore there is likely to be an additional necessity for PKCδ to work in sequence.

The serine and threonine phosphorylation response contrasts with that seen at the tyrosine site. Previously TNF has been shown to drive tyrosine phosphorylation on the junction protein, P120 in endothelial cells (Angelini *et al*., 2006) but it does not appear to cause a similar effect with CAR. There are multiple alternative cytokines that could instead drive phosphorylation of the tyrosine site and further work would need to be undertaken to establish whether this is the case. It would also be interesting to determine whether any cytokine response was cell type specific as the P120 data was confined to an endothelial cell line as opposed to the epithelial cells used in this study.

6.2 Phosphorylation of the cytoplasmic tail of CAR controls CAR-dependent leucocyte transmigration.

The necessity for CAR to be present at the cell membrane to facilitate the transmigration of leucocytes has previously been established (Zen *et al*., 2005, Witherden *et al*., 2010, Verdino *et al*., 2010). However, these previous studies have failed to show whether CAR plays an active role in controlling this movement or is simply a passive intermediary. Work presented in this thesis demonstrates that the phosphorylation of the cytoplasmic tail facilitates TEpM. These data demonstrate that the CAR cytoplasmic tail is phosphorylated during TEpM and where this is prevented the efficiency of TEpM is reduced. By so doing control of the cytoplasmic domain of CAR has an active role in TEpM thereby placing the tail in a new role in mediating the inflammatory cascade. As described, CAR is activated in inflammatory conditions and this has the added effect of controlling the physiologically relevant function of TEpM of leucocytes that is required for an ongoing inflammatory response. In so doing it emphasises the concept of the mesenchymal trophic unit in the pathogenesis of asthma (Holgate *et al*., 2000). This concept emphasises the key role played by the epithelium in the control of the pathogenic mechanism seen in asthma through direct contact of leucocytes with the inflammatory stimulus.

This data may provide an explanation for the wide range of responses to TNF α inhibitors observed in the treatment of patients with asthma. There is a known paracrine release of TNF α by transmigrating leucocytes (Finsterbusch *et al*., 2014) which is likely to cause local phosphorylation of proteins such as CAR thereby altering junctional complexes at a very local level. Given their proximity to the individual junctions when released it would be difficult to prevent their effect with the systemic anti-TNF α therapies used in current clinical studies (Erin *et al*.,

2006, Brightling *et al*., 2002, Rennard *et al*., 2007). To ensure a sufficient local response to therapy a high treatment dose would be required leading to the previously observed associated complications of systemic anti-TNF α therapy (Wenzel *et al*., 2009). However, by targeting the phosphorylation site on the cytoplasmic tail of CAR some of the inflammatory effects of TNF α may be prevented without the associated complications of inhibiting it's intended other functions. The acute mouse model supports this as a future treatment model as the use of Ad5FK was sufficient to reduce the number of neutrophils in the airway. Use of Ad5FK may not be ideal since it also reduced CAR homodimerisation and in doing so may destabilise epithelial cell junctions. An alternative might be to instead target the upstream factors in the pathway to CAR phosphorylation to overcome some of these difficulties, including PKC δ, PI3K and the TNF α/TNFR1 complex. PKC δ could theoretically provide one option as it has previously been inhibited in order to potentiate the effect of chemotherapy with significant benefits *in vivo* (Pabla *et al*., 2011). These findings though have failed to lead to a clinically effective treatment as importantly the inhibitors so far developed struggle to have specific effects on particular PKC isoforms and as a result have had many unintended off target effects (Mochly-Rosen *et al*., 2012). The targeting of the alternative kinase PI3K also has the issue of unintended off target effects. As a member of the PI3K/AKT/mTOR pathway it has multiple roles in cellular function and therefore its inhibition has been clinically unhelpful in previous trials (Rodon *et al*., 2013). Treatment of lung conditions can be undertaken via inhaled therapies, though, which reduces the systemic burden thereby reducing side effects. The mouse experiments in this thesis demonstrate that this could be used to prevent the phosphorylation CAR by administration of inhaled Ad5FK . As outlined the use of Ad5FK will lead to preferential binding to

the extracellular domain of CAR thereby preventing CAR homodimerisation *in trans* but an alternative could be to use inhaled treatments to target the respiratory system and block the binding of TNF α to its receptor with the resulting loss of CAR phosphorylation.

Identifying those patients in whom such treatment would be helpful to its resultant efficacy. Our understanding of many diseases such as asthma has significantly increased over the last decade and with it has come an increased awareness of the heterogeneity of these diseases. This is of particular relevance in this model of disease as the response from CAR seems to be confined to a limited range of cytokines and *in vivo* is specific to the function of neutrophils. Therefore future work would need access to multiple samples from patients with a variety different pathologies to determine firstly whether there is abnormal function of CAR in their conditions and to use primary airway cells to compare the response to different stimuli and treatments.

6.3 Potential mechanisms for CAR mediated TEpM.

A link between the cytoplasmic tail of CAR and its extra cellular domain has not been investigated so it remains unclear how blocking CAR homo-dimerisation might alter events at the cytoplasmic face of the cell. In particular the interaction with the extracellular domain and other members of the JAM family during TEpM has been reported as a passive process (Zen *et al*., 2005, Verdino *et al*., 2010, Witherden *et al*., 2010). Data presented in this thesis clearly demonstrate that phosphorylation of either or both the CAR serine/threonine sites results in an increase in TEpM. This may reflect a conformational change in the CAR extra cellular domain to improve its ability to heterodimerise with alternative JAM family members. TNF α has been shown to signal through phosphorylation and

conformational change of Pin1 binding to p47phox previously (Boussetta *et al*., 2010). This process of conformational change would require a significant alteration of the protein to occur across the epithelial cell membrane which is not an effect that has been specifically shown in previous studies of CAR. However, our previously published work demonstrated a band shift in the western blot analysis of CAR in response to its phosphorylation which may indicate conformation change (Morton *et al*., 2013). A conformational change in the extra cellular domain of CAR may also switch CAR from favouring homodimerisation to hetero-dimerisation with neutrophil expressed JAM-L, thereby facilitating TEpM. This process of altering the barrier function of the epithelium through the phosphorylation of a tight junction protein in order to aid its binding to other junctional proteins has been seen with occludin highlighting this as a possible mechanism (Cummins, 2012). However, to date conformational changes in CAR have not been reported.

An alternative method for this process may instead relate to its role in the junctional complex and the impact this has on stability. This response to TNF α has previously been associated with other members of the tight junction complex as occludin, claudin 1, claudin 4 and JAM-1 have all been found to internalise in detergent insoluble membrane microdomains in epithelial cells (Ivanov *et al*., 2004). We have shown that when the pSer/293 and pThr/290 sites are activated there is an alteration in the composition of the junctional complex with an associated alteration in E-Cadherin in adherens junctions (Morton *et al*., 2013). This process could in turn ensure the passage of leucocytes between epithelial cells would be easier as they would meet less resistance. This process where epithelial junctions ‘unzip’ in the presence of phosphorylated CAR to facilitate TEpM is partially contradicted by the paracellular permeability data. This has

shown that the presence of CAR reduced the known effect of TNF α on junctional permeability.

Transmigration is a controlled process requiring the leucocyte to move through the epithelium by interacting and binding to individual epithelial cells. Therefore although the junctions may not be disrupted in such a way as to cause increased solute permeability they may instead be primed to facilitate the passage of whole leucocyte cells. Additional work undertaken within the group has specifically looked at the role of threonine290 and serine293 phosphorylation of CAR in junction permeability (S. Raghavan, unpublished data). In these experiments an epithelial layer comprising either CAR-GFP cells or AACAR-GFP cells were treated with TNF α and the cell junctions imaged. Interestingly the AACAR-GFP cell junctions were maintained for significantly longer before disassociating in the presence of TNF α suggesting that although the presence of CAR may limit the impact of TNF α on epithelial barrier permeability this effect is reduced when the cytoplasmic tail is phosphorylated.

Finally, the observed increase in TEpM following phosphorylation of CAR in response to TNF α may be due to downstream cell signalling. TNF α has been shown to activate the Rho GTPase pathway leading to an alteration in the actin cytoskeleton that promotes junction permeability (Mong *et al*., 2008). Although this was undertaken in endothelial cells and led to increased permeability of the vascular system its relevance is supported by previous work from the group that showed a significant increase in Rho FRET efficiency in CAR-GFP cells when compared to wild type cells and interestingly in the AACAR-GFP mutants where the cytoplasmic tail of CAR is altered. TNF α stimulation of Rho has also been shown to drive MAPK to cause permeability changes (Nwariaku *et al*., 2003).These findings are, however, unlikely to be relevant to CAR

phosphorylation at the threonine290 and serine293 sites as the data showed that MAPK activity was unaltered by the presence or absence of phosphorylated CAR. An alternative pathway that is known to be associated with endothelial permeability and Rho activity in response to TNF is MRCK (Vandenbroucke *et al*., 2008). Although this is again focused on endothelial cells, mass spectrometry analysis of phosphorylated CAR undertaken by the group shows an association with MRCK. It would therefore be interesting to determine whether TNF induced CAR phosphorylation leads to an increase in MRCK activity or binding to CAR. However, despite findings showing that Rho and MRCK are activated in the presence of TNF their inhibition does not prevent an increase in permeability (McKenzie and Ridley, 2007). In this case loss of permeability was maintained by a loss of the tight junction proteins JAM-A and occludin at junctions which may suggest that the presence of CAR itself at the junction along with its interaction with other junctional proteins plays a more significant role than its effect on downstream signalling.

6.4 Conclusion

In summary these results are the first to show that the cytoplasmic tail of CAR is responsible for the extra cellular function of the protein and significantly that this process is activated in inflammatory conditions. Significantly, this provides CAR with a new role in the immune response that can be manipulated to the control its effect. The model system in figure 5.1 highlights the steps that have been identified in this process and importantly includes the inhibition of the pSer/293 and pThr/290 phosphorylation of CAR in the presence the Ad5FK. Given that this in turn results in the inhibition of neutrophil transmigration in the mouse model it demonstrates a novel target mechanism for possible control of inflammation in a variety of disease states.

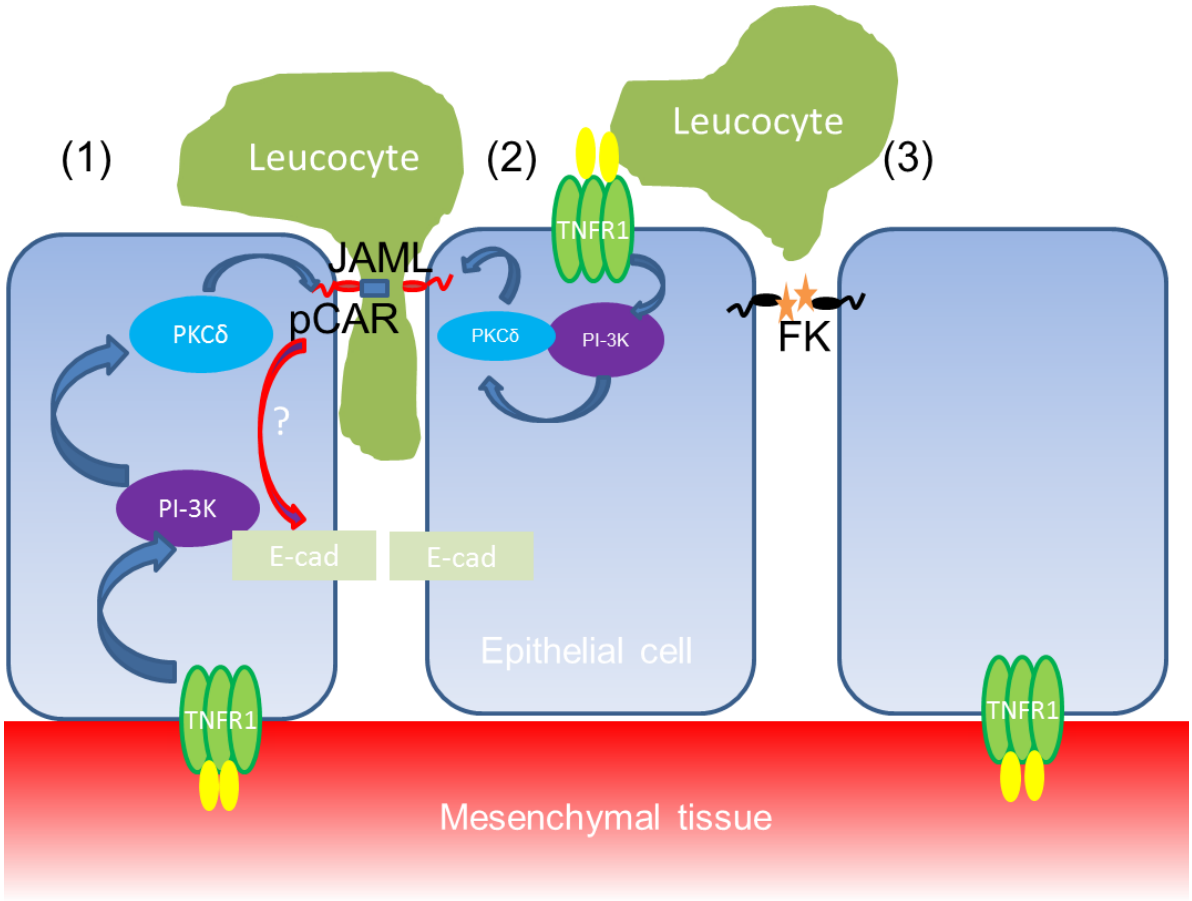


Figure 6-1: Proposed model for phosphorylation of CAR leading to transepithelial migration of leucocytes.
1) Systemic TNF binds to the TNF receptor to trigger serine/threonine phosphorylation of the cytoplasmic tail of CAR via PI-3K and PKC δ enabling leucocyte transmigration possibly through alteration of other junctional proteins.
2) The same pathway is activated by TNF release from the transmigrating leucocytes. 3) Transmigration is prevented in the presence of adenovirus fibre knob which both prevents the homerdimerisation of CAR due to competitive binding and blocks JAM-L binding to CAR.

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